NITROGEN FIXATION: FUNDAMENTALS AND APPLICATIONS

Current Plant Science and Biotechnology in Agriculture

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The titles published in this series are listed at the end of this volume.

Nitrogen Fixation: Fundamentals and Applications

Proceedings of the 10th International Congress on Nitrogen Fixation,
St. Petersburg, Russia, May 28–June 3, 1995

edited by

IGOR A. TIKHONOVICH and NIKOLAI A. PROVOROV
All-Russia Research Institute for Agriculural Microbiology,
St. Petersburg-Pushkin, Russia

VASSILY I. ROMANOV
A.N. Bach Institute of Biochemistry,
Moscow, Russia

and

WILLIAM E. NEWTON
Department of Biochemistry and Anaerobic Microbiology,
Virginia Polytechnic Institute and State University,
Blacksburg, Virginia, U.S.A.

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PREFACE

Among the most urgent problems that face Mankind as we approach the end of the 20th century are providing sufficient nourishment to the constantly growing World, reducing environmental pollution and conserving energy. The intense interest in biological nitrogen fixation shown by scientists from diverse fields of research (chemists, biochemists, molecular biologists, geneticists, microbiologists, agrochemists and many others) is obviously connected with the fact that understanding the main steps of this process will help people cope with the global problems mentioned above.

We believe that any division between fundamental and applied science is artificial; there is really only Science and its practical applications. While developing the Scientific Program of the 10th International Congress on Nitrogen Fixation, that is reflected by this volume, the Organizing Committee was always mindful of the fact that nitrogen fixation presents a broad spectrum of scientific problems of high fundamental interest while at the same time having a very significant practical value. It is important to stress that, even in the countries with traditionally high application of mineral nitrogen, interest in a sustainable balanced agriculture, and thus in the application of microbial inoculants, is growing. So it is easy to understand why this Congress had three of its 13 scientific sessions (7 Symposia and 6 Round Tables) devoted to applied aspects.

Among the many scientific aspects that were discussed in the Congress in St. Petersburg, three should be mentioned because of their active development of the past several years: (1) the impact of the nitrogenase structures on efforts to understand the mechanism that occurs in all nitrogen-fixing microorganisms; (2) dissection of the elaborate system of signal exchange between plants and microorganisms that undergirds the formation of the symbiotic apparatus responsible for the majority of biologically fixed nitrogen in agriculture; (3) our increasing knowledge about the diversity and evolution of microorganisms that are able to fix nitrogen and the attempts to systematize them thorough molecular genetic techniques. The importance of the last is difficult to overestimate - among others, this work should help to develop biological methods for protection of plants against phytopathogens. Thus, the phenomenon of biological nitrogen fixation presents a wonderful model for the study of many fundamental aspects of science in quite a number of different fields.

The 10th International Congress on Nitrogen Fixation took place in St. Petersburg, Russia, May 28 - June 3, 1995 and was organized through the All-Russia Research Institute for Agricultural Microbiology in St. Petersburg-Pushkin. The Congress was supported and sponsored by a wealth of organizations, both within and from outside

Russia, as listed elsewhere in this volume. Without their generous assistance, this Congress could not have been organized. About 600 participants from over 60 countries gathered to share their recent research progress with one another and to come to know the history and beauty of St. Petersburg. In this book, we have tried to reflect the main questions that were discussed in the Congress and to detail the contributions of every participant. We hope this volume will promote new insights, more significant research and fruitful cooperation.

We thank all those listed elsewhere who participated in the organization of the Congress, its smooth day-to-day operation, and the preparation of this volume. Lastly, we thank Vicki Newton for her tireless efforts to keep us on our tight publication schedule and our families and friends for their love, patience, support and understanding during this intense experience.

Saint - Petersburg,
Russia, June 1995

Igor A. Tikhonovich
Nikolai A. Provorov
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SECTION 1

INTRODUCTORY AND OVERVIEW LECTURES

CHEMICAL NITROGEN FIXATION IN SOLUTION

VOLPIN M.E.(1) AND SHILOV A.E.(2)
(1) Institute of Organometallic Compounds, Russian Academy of Sciences, Moskow.
(2) Institute of Chemical Physics in Chernogolovka, Moskow region, Russia.

Introduction

Both biological nitrogen fixation and chemical synthesis of ammonia from dinitrogen and dihydrogen are of great significance for human life. Up to the present time, bacteria (free-living in the soil and in nodules in symbiosis with plants) perform the main part of nitrogen cycle in nature, and the industrial synthesis of ammonia provides people with necessary nitrogen-containing substances (fertilizers, plastic materials, dyes, medicaments, etc.).

These processes are similar in stoichiometry (ammonia is formed primarily in the enzymatic process), but their conditions are quite different: According to the known chemical inertness of dinitrogen, the catalytic synthesis of ammonia occurs under high pressures and temperatures, while the biological nitrogen fixation occurs under ambient conditions. For a long time (up to the middle sixties of our century), virtually no low-temperature dinitrogen chemistry existed, and the biological nitrogen fixation aroused the curiosity of chemists who tried (unsuccessfully) to reproduce it under non-biological conditions. The question concerning the way in which the inertness of dinitrogen is overcome during the enzymatic reduction remained unanswered.

At present, the situation has changed drastically. Now we know a number of low-temperature reactions of N_2: its reduction in solution to metal nitrides, organonitrogen compounds, hydrazine and ammonia (both stoichiometric and catalytic), formation of complexes of N_2 with metal-containing compounds of various types, and chemical transformations of the N_2 molecule in these complexes.

Dinitrogen reduction in aprotic media

Table 1 presents the chronology of the main events in dinitrogen chemistry beginning from the discovery of first reactions in solution in 1964 by Volpin and Shur and discovery of the first complexes of N_2 with bivalent ruthenium compounds by Allen and Senoff.

The first reactions of dinitrogen reduction required strong reducing agents and occurred only in aprotic media in the presence of transition metal compounds. In the majority of cases, the yield of ammonia (after the hydrolysis of products) is not greater

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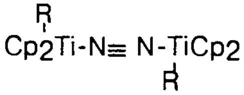
Table 1

First events in the history of N_2 reduction in solution

Year	Reaction	Autors
1964	$N_2 + 6e \xrightarrow{Red,MX_n} 2N^{3-} \xrightarrow{H^+} 2NH_3$ Red= $LiAlH_4$, RMgX, *i*-Bu_3Al, Mg-MgI_2 etc. MX_n= $TiCl_4$, $CrCl_3$, $MoCl_5$, WCl_6, $FeCl_3$, etc.	M.E. Vol'pin V.B. Shur
1965	$RuCl_3 + N_2H_4 \xrightarrow{I^-} (NH_3)_5Ru(N\equiv N)I_2$	A.D.Allen C.V. Senoff
1966	$RuCl_3 + N_2 + Zn(Hg) \xrightarrow[THF]{} (THF)_3Ru(N_2)Cl_2$	A.E. Shilov A.K. Shilova Yu.G.Borod'ko
1966	$Cp_2TiPh_2 \xrightarrow{N_2} [Ti-N=N-Ph] \xrightarrow{H^+} PhNH_2 + NH_3$	M.E. Vol'pin V.B. Shur
1967	$[(NH_3)_5RuN_2]^{2+} + [(NH_3)_5Ru(H_2O)]^{2+} \xrightarrow[-H_2O]{}$ $[(NH_3)_5Ru-N\equiv N-Ru(NH_3)_5]^{4+}$	D.E. Harrison H. Taube
1969-1971	$Cp_2TiCl_2 + RMgX + N_2 \longrightarrow$  $Cp_2\overset{R}{Ti}-N\equiv N-\underset{R}{Ti}Cp_2$ $\xrightarrow[Red,\ H^+]{} N_2H_4$	A.E. Shilov A.K. Shilova E.F. Kvashina J.H. Teuben et al.
1970	$N_2 + 4e + 4H_2O \longrightarrow N_2H_4 + 4OH^-$ $N_2 + 6e + 6H_2O \xrightarrow{Red,MX_n} 2NH_3 + 6OH^-$ **MX_n = VCl_2, $MoCl_5$**	N.T. Denisov V.F. Shuvalov N.I. Shuvalova A.K. Shilova A.E. Shilov
1973-1977	*cis*-$[M(N_2)_2(PR_3)_4] + H_2SO_4 \longrightarrow$ $\rightarrow N_2 + 2NH_3 + M(VI)prod + 4[PR_3H][HSO_4]$ $\rightarrow N_2 + NH_3 + M(III)prod$ M = W, Mo, PR_3 = PMe_2Ph, $PMePh_2$	J. Chatt A.J. Pearson R.L. Richards

than the stoichiometric yield calculated per transition metal compound. Complex nitrides are usual reaction products. Some of the systems studied turned out to be very active and capable of reducing dinitrogen at room and lower temperatures and pressures.

In addition to these systems, catalytic systems with such reducing agants as Al-$AlBr_3$ or $LiAlH_4$-$AlBr_3$ were also discovered. In these cases, tens and sometimes hundreds of moles of nitride per mole of the transition metal used can be obtained. Although no catalytic hydrogenation was observed in these systems (dihydrogen usually acts as an inhibitor), in several cases the yield increased in the presence of H_2 and it was found to participate in the reduction. In some systems, organonitrogen compounds, for example, aniline and aniline derivatives, were formed.

Dinitrogen reduction in protic media

Beginning in 1970, dinitrogen reduction in protic media, mainly methanol and water, involving relatively weak reducing agents have been discovered. For the most part, the systems discovered were heterogeneous, for example, they included metal hydroxides $V(OH)_2$-$Mg(OH)_2$ or $Mo(OH)_3$-$Ti(OH)_3$. One of the systems, complexes of V^{II} and catechol in methanol or water solutions, turned out to be homogeneous. The products are hydrazine and (or) ammonia. The compounds of V^{II} and Mo^{III} are the most active (the latter required the presence of additional stronger reducing agent). Then the systems involving Nb^{III}, Ta^{III}, Ti^{II}, and Cr^{II} were found which could also reduce N_2 in protic media. In the case of Mo^{III} complexes the catalytic reduction is observed in the presence of a stronger reducing agent, for example, $Ti(OH)_3$, or sodium amalgam.

On the mechanism of dinitrogen reduction. The reasons for N_2 inertness and the methods to overcome it.

The theory, which allows us to understand what properties should be inherent to the systems reducing dinitrogen with mild reducing agents, was created at the moment of the discovery of dinitrogen-reducing systems in protic media. Consideration of the thermodynamics of the consecutive cleavage of the three bonds of dinitrogen gives a clue to the solution of this problem. Although the triple bond in the N_2 molecule is rather strong (225 kcal/mol), its dissociation energy is almost the same as that of the triple bond in acetylene, while the bond in CO is even stronger. The inertness of the N_2 molecule is determined by the strength of the first breaking bond, which corresponds to about a half of the whole energy of the triple N=N bond in dinitrogen (ca. 100 kcal/mol) and is considerably stronger than the first breaking bond in acetylene (ca. 60 kcal/mol). On the contrary, the second and third breaking bonds that are cleaved in dinitrogen, are rather weak, and this makes it thermodynamically possible to use a relatively mild reducing agent for complete or partial bond breaking in N_2 to form ammonia or hydrazine, respectively. This can be performed using the four- or six-electron reduction of N_2, which does not require too strong a reducing agent. The formation of a binuclear complex M-N=N-M allows this reaction to proceed in one stage. In this case, the filled d-orbitals of the M metal atoms interact simultaneously with both antibonding π_g orbitals of dinitrogen. The protonation of this complex results in the formation of a hydrazine derivative

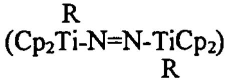
$$\mathrm{M{-}N{=}N{-}M} + \mathrm{H^+} \longrightarrow \mathrm{M{=}N{-}\overset{+}{N}H{=}M}$$

It is clear that neither diazene nor its derivative are formed in this process as intermediates. Thus, the term "four-electron" mechanism is close to the physical essence of this process. Further analysis shows that the reaction can generally be facilitated when the binuclear complex is included in a cluster that consists of several metal atoms each being capable of at least one-electron donation.

Qualitative consideration of the molecular orbitals of the complex lead to the conclusion that compounds of metals (M) with the d^2 and d^3 electron configurations should be optimum catalysts. Thus, the observed activity of the polynuclear compounds of vanadium(II) and molybdenum(III) as well as niobium(III), tantalum(III), and titanium(II) is natural and corresponds to the suggested mechanism. We succeeded in isolating polynuclear complexes and determining the structures for some of the systems. Thus, it is certain that the active complexes are polynuclear in the systems, which reduce dinitrogen in protic mediain. The octanuclear complex of $\mathrm{Mo^{III}}$ is an efficient catalyst for dinitrogen reduction in the complex catalytic system, which contains sodium amalgam (or mercury cathode) as a reducing agent, a surfactant (for example, phospholipid or polyvinyl alcohol), and additives of phosphines. In the full system, the specific reaction rate calculated per active complex is even greater than the rate of enzymatic dinitrogen reduction. The number of catalytic cycles can be several thousands. However, it should be noted that a much stronger reducing agent than in the natural system is used. Thus, the inertness of dinitrogen is relative. Dinitrogen becomes highly reactive in the case of a polynuclear complex in which N_2 can undergo multi-electron reduction.

Dinitrogen complexes. Conclusion about nature of intermediate complexes in N_2 reduction.

The structures of intermediate complexes remained unclear for a long time. After the discovery of the first intermediate binuclear complexes between N_2 and $Cp_2Ti(III)R$,



$$(\overset{R}{\mathrm{Cp_2Ti}}\mathrm{{-}N{=}N{-}}\underset{R}{\mathrm{TiCp_2}})$$

the mononuclear complexes of W° and Mo° of the $(R_3P)_4M(N_2)_2$ type in which dinitrogen is reduced to ammonia or hydrazine under the action of an acid became very famous. Since other types of complexes with reducible dinitrogen were virtually unknown, the tendency was to consider such mononuclear complexes as the intermediates in dinitrogen reduction reactions in solution, including enzymatic dinitrogen reduction. One should note that the initial states of metals in these complexes correspond to low valencies, and the dinitrogen reduction in the case of $\mathrm{W^0}$ results in its oxidation to $\mathrm{W^{VI}}$ to form two NH_3 molecules, which, of course, cannot be suitable for a catalytic system with a weak reducing agent.

Moreover, later (mainly, after the eighties), binuclear linear complexes between N_2 and compounds of $\mathrm{V^{II}}$, $\mathrm{Mo^{IV}}$, $\mathrm{W^{IV}}$, $\mathrm{Nb^{III}}$ and $\mathrm{Ta^{III}}$, in which dinitrogen can be reduced, were synthesized. The metals (and their oxidation states) that are active in the systems reducing nitrogen in protic media (series 1), may be compared with the metals that form binuclear complexes with dinitrogen and can reduce the bound dinitrogen to hydrazine or ammonia on protonation (series 2).

(1)	$Ti^{II}(d^2)$		$V^{II}(d^3)$	$Nb^{III}(d^2)$	$Ta^{III}(d^2)$	$Cr^{II}(d^{IV})$	$Mo^{III}(d^3)$	
(2)	$Ti^{II}(d^2)$	$Zr^{II}(d^2)$	$V^{II}(d^3)$	$Nb^{II}(d^2)$	$Ta^{III}(d^2)$		$Mo^{IV}(d^2)$	$W^{IV}(d^2)$ $W^{II}(d^4)$

A close coincidence can be seen and this confirms the conclusion that the linear complexes of the M-N=N-M type with the d^2, d^3 or sometimes d^4 electronic configurations of M can really be very likely intermediates in dinitrogen reduction in protic media.

It looked reasonable to expand this conclusion to biological nitrogen fixation, where two Mo atoms are known to be present in the MoFe protein or two V atoms in the vanadium nitrogenase, which activates dinitrogen. However, this conclusion turned out to be wrong. Although the suggested multi-electron mechanism of dinitrogen fixation is almost certainly correct, the mechanism involving the linear binuclear M-N=N-M complex apparently does not cover all possible cases.

The data indicating non-linear structures of intermediate complexes, which, nevertheless, also use the multi-electron mechanism to reduce N_2 appearred recently. It turns out that complexes with side bonding to metal ions also exist. The necessity of four electrons for N_2 activation is especially pronounced in the case of these complexes. One or two electrons virtually do not change the N-N bond length, while four electrons decrease the multiplicity to a single bond (the N-N bond is even longer than that in hydrazine). It is demonstrated for several cases that the activation of the N_2 molecule is especially significant for multiple coordination. Both theory and experiment lead to the conclusion that the d^2-d^4 electron configuration is necessary for the linear complex, while these restrictions are not evident for non-linear complexes and are probably non-existent.

The results on the structure of the FeMo-cofactor not only indicate that the intermediate complex of the MoNNMo or VNNV types is impossible, but also makes the mechanism in which iron is the only metal directly contacting dinitrogen very probable. Although this conclusion is not final, the coordinative unsaturation of the iron atoms, the appropriate interatomic distances between the iron atoms to coordinate dinitrogen and at the same time coordinative saturation of the molybdenum atom makes iron the likely candidate to coordinate dinitrogen in the polynuclear complex.This is also indicated by the existence of the "iron-only nitrogenase". The fact that this nitrogenase is extremely unstable confirms the idea that the participation of molybdenum (or vanadium) only stabilizes the complex without substantial changes in its reducing properties. In this connection, it is natural to anticipate the existence of efficient catalytic systems, which involve only iron as a transition metal. The discovery of such systems and demonstration of their high activity is the necessary (albeit insufficient) condition to validate the conclusion of an iron active center in nitrogenase.

Dinitrogen-fixing systems involving iron atoms

It is reasonable remember the Haber catalysts, which in industrial systems are based on iron modified by various additives without other transition metals. According to the present views, there is a side coordination of dinitrogen in the intermediate complex. Of course, it should be realized that iron functions in the Fe^{o} state in the catalyst, but although iron of the FeMo-cofactor in the active complex does not decrease its oxidation state to zero, there are sulfide bridges that can serve as additional donors, plus the participation of protons can and should activate N_2 upon its protonation. Let us also remember that one of the weakest possible reducing agents, dihydrogen, participates in the industrial catalytic process. It turns out that the catalysts based on iron (or ruthenium and osmium) for the synthesis of ammonia can be activated by adding active electron donors, for example, the alkaline metals, potassium, rubidium, or cesium. This allows a considerable decrease in reaction temperature and pressure. For example, a stable high rate of dinitrogen hydrogenation at 300^0 to 350^0C is observed in the system based on iron deposited on activated carbon and modified by potassium. The participation of these additional reducing agents can play a role similar to that of the double P-cluster in the MoFe-protein of nitrogenase. This reducing agent facilitates the transformation of the complex into the hydrazine derivative, which probably determines the rate of the whole process.

Iron complexes in aprotic media are also active in the Volpin-Shur reactions involving strong reducing agents. It is evident that nitride derivatives are formed in this process to yield ammonia in the decomposition by an acid. The stronger the reducing agent, the higher is the yield of ammonia per iron. However, no catalytic yields are observed in solution. The reaction between N_2 and the tetraphenyl-trans-dihydride iron lithium complex in THF results in the formation of the binuclear hydride iron(II) complex with N_2, which can be partially reduced by protons to form hydrazine and ammonia. Dinitrogen is reduced in methanol to form ammonia when a strong reducing agent (lithium-polyphenylene) reacts with iron compounds.

All these data demonstrate the possibility of activating nitrogen by iron compounds under mild conditions. However, catalysts based on iron-sulfur clusters to catalytically reduce nitrogen by weak reducing agents have not yet been reported. The polynuclear molybdenum complex described above is the most active catalyst so far for both dinitrogen and acetylene reduction at room temperature. Nevertheless, the distinctions in activity observed for iron and molybdenum complexes are quantitative and not qualitative. It can be assumed that further development in the synthesis of new iron-sulfur clusters (perhaps, with the participation of molybdenum or vanadium) will help to create new catalytic systems, whose structures and activities are similar to those of the nitrogenase cofactor.

THE MOLECULAR DEVELOPMENT OF NITROGEN FIXING ROOT NODULES

VAN KAMMEN, A.
Dept. of Molecular Biology, Wageningen Agricultural University,
The Netherlands.

Introduction

Biological nitrogen fixation is the enzymatic reduction of dinitrogen (N_2) from the atmosphere, to ammonia. The ability to fix nitrogen is only found in a variety of prokaryotic organisms, but not in eukaryotes. The nitrogen fixing bacteria have the enzyme system, nitrogenase, which provide the biochemical machinery for nitrogen fixation. Some nitrogen fixing bacteria like *Klebsiella pneumoniae*, *Azotobacter* and *Rhodopseudomonas* are able to reduce nitrogen into ammonia as free living organisms. Other bacteria fix nitrogen actually only in symbiotic relationships with an eukaryotic host plant, like rhizobia in legume symbiosis, *Frankia* in actinorhizal symbiosis and cyanobacteria in *Gunnera* symbiosis. The best studied examples of symbiotic nitrogen fixation are the soil bacteria of the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*, collectively referred to as rhizobia, which have the ability to induce the formation of nodules on the roots of leguminous plants. In these nodules the bacteria are able to fix nitrogen and provide the host plant with ammonia for plant growth. The symbiotic nitrogen fixation reduces the need for N fertilizer and the legume crops have a high nutritive value and enrich the N content of the soil. Therefore, symbiotic nitrogen fixation is of great importance to agriculture. The legume-*Rhizobium* association is now an intensively used model system for studying the mechanism by which rhizobia induce nodule formation and the factors which determine effective symbiotic nitrogen fixation by plants.

The overall stoichimetry of the biological nitrogen fixation reaction

$$N_2 + 8H^+ + 8e^- + 16\,MgATP \xrightarrow{\text{nitrogenase}} 2NH_3 + H_2 + 16MgADP + 16Pi$$

indicates the requirements for reducing equivalents, MgATP and protons for the nitrogenase-catalyzed reactions. In aerobic bacteria like *Rhizobium*, the large amount of MgATP has to be generated by oxidative processes. On the other hand, the bacterial nitrogenase is highly oxygen sensitive and will only function at low oxygen concentration in a microaerobic environment. It is an intriguing problem of the physiology of root nodules how the nitrogen fixing system of the bacteria in nodules copes with this problem.

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In the following I shall comment on some aspects of the formation of symbiotically nitrogen fixing root nodules, the functioning of nitrogenase and the regulation of nitrogen fixing activity in nodules to explain why this research holds a prominent position in plant molecular biology and biochemistry.

Root nodule formation in the Rhizobium-legume symbiosis

The interactions of legumes and *Rhizobium* result in the formation of root nodules - new organs in which the bacteria are able to fix atmospheric nitrogen into ammonia. Specific flavonoid compounds, secreted by the plant root, activate the bacterial *nodD* gene to produce a transcriptional activator for the expression of other *nod* genes. These *nod* genes are responsible for synthesis of mono-N-acylated chitin oligomers, called Nod factors, that function as signalling molecules which trigger nodulation in the host plant. All rhizobia produce Nod factors which belong to the same family of lipo-oligosaccharides, but host specific *nod* genes specify the modification of the Nod factors to make them plant-specific. Modifications found in Nod factors include sulfation, glycosylation, acetylation and variation in the length and in the number of unsaturated bonds of the acyl chain (Denarie, Cullimore, 1993; Spaink, 1992).

The development of a nodule starts with the attachment of rhizobia to the root hairs of their host, which causes deformation and curling of root hairs followed by the formation of infection threads in the curled hairs. The bacteria enter the root through these threads. Concomitantly with the infection process, the bacteria induce cell divisions in the root cortex, which leads to primordium formation. The infection threads grow towards the centres of mitotic activity, then enter primordium cells, and bacteria are released into the plant cells by an endocytotic process. Then the nodule primordium differentiates into a mature nodule, which provides the proper environment for the bacteria to fix nitrogen (Hap, Bisseling, 1990).

Each of the successive steps in nodule formation is marked by the expression of nodule-specific plant genes that code for plant proteins called nodulins. The nodulin genes that are expressed before the onset of N_2 fixation are named early nodulin genes. The nodulin genes expressed shortly before, or at the same time as N_2 fixation are the late nodulin genes. Examples of early nodulin genes are ENOD5, ENOD12 and ENOD40 (Franssen et al. 1992). *In situ* hybridization studies have revealed the expression pattern of each of these early nodulin genes. After inoculation of pea roots with *Rhizobium leguminosarum*, ENOD12 is induced in root hairs, root cortical cells containing growing infection threads, in nodule primordium cells and in nodule cells containing growing infection threads. On the other hand, the ENOD5 gene is expressed only in cells containing the growing infection thread (Schtres et al. 1990). ENOD40 is activated in all cells of the primordia just as ENOD12, but also in the region of the pericycle opposite to the dividing cortical cells. The spatial pattern of expression of ENOD5, ENOD12 and ENOD40 is thus strikingly different and the expression of each of these nodulin genes marks specific early steps of nodule development.

An important observation is that purified Nod factors induce a spatial pattern of early nodulin expression which precisely corresponds to the pattern after *Rhizobium* infection. Nod factors, in the absence of bacteria, are able to induce cell divisions in the inner cortex of the root and early nodulins genes ENOD12 and ENOD40 are expressed in such primordia (Horvath et al. 1993; Vijn et al. 1993). In addition

ENOD40 gene expression is activated in the root pericycle facing the dividing cells, opposite to proto-xylem poles. So Nod factors induce cortical cell divisions as well as nodulin gene expression in a spatially controlled manner (Vijn et al. 1993; Yang et al. 1994). How the Nod factor signalling system effects the well-defined pattern of gene expression in still to be analyzed. The Nod factor molecules that induce the initial steps of nodulation have a very specific structure and are active at nanomolar to picomolar concentrations. This strongly suggests that they act through receptors. The nature of this possible receptor-Nod factor interaction is not yet known. Likewise, with respect to signal induction, the question remains to be answered whether Nod factors, or the active parts of Nod factors, are transported by a specific mechanism towards the inner cortical cells. On the other hand, it is equally possible that secondary signal molecules elicited in the epidermis of the root are involved. The changes in gene expression in roots induced by *Rhizobium*-bacteria and purified Nod factor constitute a favourable system for analyzing these fundamental questions of signal transduction.

Recently, evidence has been obtained that Nod factors can also influence development in a non-legume plant (De Jong et al. 1993). It was shown that a mutant cell line from carrot, which is arrested in somatic embryogenesis, can be rescued by addition of Nod factors. Since carrot is not a legume, and the cells of the carrot cell line, would not in any case be involved in nodulation, this result might mean that Nod-type molecules play a role in normal plant development. Futhermore, Nod-factor recognition mechanisms might occur in non-legume plants. If plant produced Nod-factor-like molecules can be involved in non-symbiotic developmental processes in plants these processes might be the evolutionary origin of the bacterial signal molecules produced by *Rhizobium*. Research originally initiated to elucidate the development of nitrogen-fixing root nodules might then provide insights into aspects of plant development not recognized before.

Functional Root Nodules

When *Rhizobium* bacteria have been released from the infection threads into the plant cells, nodule primordia differentiate into nitrogen fixing nodules. Depending on their growth pattern, indeterminate and determinate legume nudules are distinguished. In both cases, legume nodules are characterized by peripheral vascular bundles and a central tissue, containing infected and uninfected cells. In indeterminate pea nodules, the apical meristem differentiates continuously and a developmental gradient from the distal persistene meristem to the proximal senescence zone is present, by which the central tissue can be divided into specific zones (Vasse et al. 1990). Nodule meristem (zone I) can be distinguished from the prefixation-zone II, where infection of the cortical cells takes place. In the interzone II-III bacterial nitrogen fixation is induced and proceeds throughout the nitrogen fixation zone III. In the senescence zone IV, bacteroids are degraded by the plant. In determinate nodules the nodule meristem ceases to divide at an early stage of development and, as a result, the cells of the central tissue are at a similar stage of development at a given time.

Using *in situ* hybridization, the expression of early and late nodulin genes, and also bacterial genes, has been studied in the zones following one another in the central tissue of indeterminate pea root nodules (Yang et al. 1993). It was demonstrated that the transition of each zone into the next is accompanied by the expression of specific nodulin genes, as well as bacterial genes, which mark different stages of development. The expression of the first late nodulin gene, the gene for leghaemoglobin starts in the infection zone II and reaches its maximum in the N_2 fixation zone III. At the transition of infection zone II to zone III, the *nif* genes of *Rhizobium* are induced in the bacteroids and in zone III the proper environment is established to allow efficient N_2 fixation by the bacteroids.

Regulation of nitrogenase activity

The nitrogenase enzyme system consists of two component proteins, the iron (Fe-) protein and the molybdenum-iron (MoFe-) protein that together are responsible for the ATP dependent reduction of N_2 from the atmosphere. The catalytic reduction of N_2 by nitrogenase involves the reduction of the Fe-protein by electron transfer carriers such as flavodoxin or ferredoxin, the ATP dependent transfer of single electrons from Fe-protein into MoFe protein, with a minimal requirement of two molecules of ATP hydrolyzed per electron transferred and, finally, electron and protein transfer to the N_2 substrate, which is bound to the FeMo-cofactor of MoFe-protein. It was a milestone in nitrogenase research when in 1992 the three-dimensional crystallographic structure of both the Fe-protein and the MoFe-protein were published (Kim, Rees 1992a; Kim, Rees 1992b; Georgiadis et al. 1992). With the knowledge of these structures a molecular level description of N_2 reduction by nitrogenase comes within reach. Above all it will allow understanding of the N_2 binding to the MoFe protein and the interaction with the FeMo cofactor which results in the so remarkable reduction of N_2 to ammonia at atmospheric pressure and room temperature.

Here I shall restrict myself to some comments on the Fe-protein and the roles of the Fe protein and MgATP in nitrogenase function. When the crystallographic structure of the Fe protein was determined, it was immediately noted that the structure of this ATP binding component had striking similaities with other nucleotide binding proteins, such as H-ras p21 and other members of the GTP-ase superfamily (G proteins), which play an important role in the regulation of a diversity of cellular processes (Georgiadis et al. 1992; Wolle et al. 1992). The striking similarity has been used to describe the function of the Fe protein as a molecular switch that cycles through two conformational states. The rate of the nitrogenase reaction is then determined by the rate by which the Fe protein goes through the cycle. The reduced Fe-protein can bind two ATP molecules, which produces a change in conformation. Upon binding to the MoFe protein, the bound ATP is hydrolysed, which effects a conformational change in the MoFe protein and electron transfer to the MoFe-protein. The Fe protein, that is now in the ADP-bound inactive conformation, dissociates from the complex of the two components and, after reduction and exchange by ATP of its bound ADP, it can pass again through the cycle. After six such cycles, the MoFe protein will have collected the electrons required for the reduction of N_2 into NH_3. The rate of the cycle, and the reduction of N_2, is virtually determined by the availability of reducing equivalents for the reduction of the Fe protein, assuming ATP is enough.

The elucidation of the three-dimensional structure of the nitrogenase components has provided the understanding that the activity of nitrogenase is regulated by the cycle of the Fe protein in which exchange of ADP for ATP turns on the switch (active Fe-protein) and ATP hydrolysis turns it off (inactive Fe-protein). As far as that is concerned the Fe protein belongs to the class of nucleotide binding proteins, such as G proteins, that regulate cellular processes as diverse as transmembrane signalling, the control of cell proliferation, photosynthesis and membrane transport by switching between alternate conformational states.

Regulation of nitrogenase activity in the bacteroids in root nodules

The nitrogenase enzyme system is very oxygen sensitive and nitrogenase is irreversible inhibited by O_2. On the other hand, there is a high demand for O_2 in nodules as the reduction of N_2 requires large amounts of energy, which is generated by oxidative phosphorylation. So, in the nodules, bacteroids must be maintained in a low O_2 environment, but supplied with a high O_2 flux. In legume nodules, a low oxygen tension in the central tissue of the nodule is achieved by a combination of a high metabolic activity of the microsymbiont and an O_2 diffusion barrier in the parenchyma in the periphery of the nodule (Witty et al. 1986). In this tissue, nodulin genes like ENOD2 are expressed and their protein products might contribute to the construction of the oxygen barrier (Van der Wiel et al. 1990). Besides, in the infected cells of the central part of the nodule, large amounts of leghaemoglobin are present and bind O_2 reversibly with high affinity. In addition to keeping the O_2 tension low in the infected cells, leghaemoglobin also facilitates the O_2-flux to the bacteroids (Appleby 1984). In this way the bacteroids are provided with O_2 to generate metabolic energy within a low overall O_2 concentration. At the low O_2 tension in the nodule, which can be as low as approx. 10nM, the bacteroids must use the available O_2 very efficiently.

Respiration of the bacteroids involves the oxidation of NADH, generated metabolically, coupled to the phosphorylation of ADP. The ATP generated can then be used by nitrogenase for nitrogen fixation. The rate of O_2 consumption is determined by the level of ADP in the bacteroids. During nitrogen fixation, nitrogenase is the large-scale consumer of ATP and that ATP consumption regulates the respiratory activity. Very recently, it has been demonstrated (Haaker et al. 1995) that bacteroids have respiratory control similar to respiratory control in mitochondria of eukaryote organisms. In their study of the relationship between the O_2 consumption rate of *Rhizobium leguminosarum* bacteroids, the intracellular ATP/ADP ratio and the whole cell nitrogenase activity, it was observed that inhibition of nitrogenase by excess O_2 increased the intracellular ATP/ADP ratio. The increase of ATP/ADP ratio is very rapid when the nitrogenase activity is inhibited more than 50%. Nitrogenase, at the O_2 input rates used (60-80 mmol O_2/min^{-1}, mg^{-1} protein), was inactivated reversibly, and not irreversibly, as full activity of nitrogenase could be recovered. It appeared that, in the O_2 concentration range in which nitrogenase activity was inhibited and the ATP/ADP ratio rapidly increased, the respiration of the bacteroids at low O_2 concentration was decreased. It appears that respiratory control functions in the bacteroid. Respiratory control by the bacteroids, and control of the nitrogenase activity by respiration of the bacteroid, was further proven by using the proton ionophore, carboxyl cyanide 4m-chlorophenylhydrazone (CCCP), as an uncoupler of respiration and phosphorylation. By adding CCCP to bacteroids at an inhibitory O_2 input, the ATP/ADP ratio was lowered and nitrogenase activity was stimulated. Respiration control as an important factor in the regulation of nitrogenase activity has so far hardly been considered. It demonstrates how ATP consumption, through ATP hydrolysis by nitrogenase, is of vital importance for the functioning of the bacteroid as a N_2 fixing organelle.

Conclusion.

The large biological significance of nitrogen fixation and the importance of the process for agriculture is prompting a major effort of fundamental research in this area. We have to understand the mechanisms of nitrogen fixation and of the regulation of nitrogen fixing activity in root nodules and other symbioses to be able to develop more effective systems and to make optimum use of biological nitrogen fixation in agriculture. The brief discussion of root nodule development, the functioning of nitrogenase and the regulation of N_2 fixing activity in root nodules illustrates the promising progress made in the fundamental research. At the same time, these investigations on symbiotic nitrogen fixation are important models of research in plant developmental biology, structural biology and molecular physiology, that occupy a prominent position in plant science.

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CONTRIBUTION OF RUSSIAN SCIENTISTS TOWARDS SOLUTION OF BIOLOGICAL NITROGEN FIXATION PROBLEM

ROMANENKO, G.A.
Russian Academy of Agricultural Sciences, Krzhizhanovskogo str. 15,
Moscow, Russia

Biological nitrogen fixation is a problem of the highest priority in biological and agricultural sciences in many countries of the world and on all continents.
The Academy of Agricultural Sciences of Russia is, by the decree of President of Russian Federation, a coordinating and methodological centre for agricultural science in Russia. It has traditionally supported the development of not only applied research on biological nitrogen fixation but also those fundamental studies which make it possible, together with the use of modern research techniques, to create novel highly effective symbiotic systems which form the basis of modern ecologically safe inoculant preparations. These preparations mark the beginning of a new era in agricultural production, namely - setting up ecologically clean agricultural production. To a great extent, it is based on optimizing basic processes occurring in agrophytocoenosis. Development of this trend is a response to ecological and energy problems which agriculture has recently encountered. An increase in research activities in the area of nitrogen fixation is to a large measure associated with a constantly increasing application of mineral fertilizers, the production of which is a power consuming and expensive process. Moreover, effectiveness of the mineral nitrogen applied to the soil does not exceed 40-50% as a rule, whereas the rest is washed into basins or having undergone various chemical changes becomes one of the major factors of environmental pollution.

The study of biological nitrogen fixation in Russia has an extensive historical background and is based on the traditions of a number of domestic scientific schools. As far back as in 1866 M.S.Voronin (1866) demonstrated that the nodules of the legumes contain bacterial cells. In 1893 S.N.Winogradsky (1952) for the first time clearly proved that nitrogen fixation was of microbiological origin. In the early thirties S.P.Kostychev founded in Leningrad (St.Petersburg) an Institute of Agricultural Microbiology which since that time and up to now is the main centre in Russia where research is conducted on biological nitrogen fixation. The work of such outstanding scientists as V.P.Israilsky (1933), E.N.Mishustin (1971), V.L.Kretovich (1972) and L.M.Dorosinsky (1970) served as a theoretical foundation for studying nitrogen fixation in Russia, for organizing large scientific centres and for creating a system for introducing nitrogen fixing preparations into agricultural production.

The All-Russia Soybean Research Institute has conducted an all-round evaluation of the collection of Far East varieties of soybean and selected those which hold potential for breeding. Wide ranging research is conducted on breeding and selection of leguminous

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crops having an enhanced potential for fixing nitrogen. The scientists of the All-Russia Research Institute for Leguminous and Groat Crops have created regional varieties of pea which accumulate twice the amount of nitrogen as compared with the former varieties. Similar results have been obtained for fodder legumes at the All-Russia Fodder Research Institute named after V.P. Williams, at the Siberian Fodder Research Institute and at the All-Russia Lupine Research Institute.

The All-Russia Research Institute for Agricultural Microbiology has selected nodule bacteria for many varieties of legumes. On the basis of multi-year competition tests on nitrogen fixing microorganisms held in the Geographic Experimental Net the largest national collection of nodule bacteria (over 700 strains for all basic legumes grown in the world) has been created. It is a source of highly effective strains for all inoculum producers. The All-Russia Soybean Research Institute has accumulated a collection of soybean nodule bacteria (over 200 strains) which possess quite a few high performance features useful for agricultural production (effectiveness, competitiveness, adaptability to production process). Among them there are fast growing soybean nodule bacteria strains effective for the varieties grown in the Far East.

In addition to traditional breeding methods wide scale research involving molecular-genetic techniques has been organized. The aim of these activities is creation of symbiotically superproductive nitrogen fixing systems. The research has resulted in highly effective strains of nodule bacteria for inoculating alfalfa, pea and vetch, which considerably surpass 'wild' strains. Joining efforts with large Research Centres of the USA, Netherlands, Germany and other countries, the All-Russia Research Institute for Agricultural Microbiology is conducting research on the delicate interrelations between plants and microorganisms occurring during the period preceding formation of nitrogen fixing systems. The analysis of a number of regulatory genes has been conducted up to now and previously unknown genes of plants and microorganisms, which determine the formation and functioning processes of symbiotic systems, have been found.

The scientists of the All-Russia Research Institute for Agricultural Microbiology have isolated from different soils of the world and rhizosphere of plants the microorganisms capable of fixing air nitrogen in association with non-leguminous crops. All-round research conducted for many years resulted in creation of a new generation of microbial biopreparations possessing a wide range of activity and multifunctional useful properties. These microorganisms not only fix nitrogen (70-80% of nitrogen fixed remains in soil) but also improve mineral nutrition and water balance of plants, promote their growth and development, suppress development of diseases, reduce accumulation of heavy metals and radionuclides in the plants. It should be noted that a number of novel preparations have no analogues abroad.

The ecological aspects of agriculture have initiated the development of a scientific basis for the wide application of biological fixation of atmospheric nitrogen by microorganisms as a way to decrease the doses of mineral fertilizers and minimize the deficit of fodder protein. Put in this way, the problem of nitrogen fixation is not of inferior significance to, for instance, the problem of photosynthesis. But our ability to regulate it is certainly greater. Substantiated expectations to save no less than 2 million tons of mineral nitrogen fertilizers and to obtain additionally up to 5 million tons of full value protein in Russia are associated specifically with an increase in the share of biologically fixed nitrogen in the yield of cultured crops. Biologically fixed nitrogen will, certainly, never replace completely the employment of mineral nitrogen, however a rational application of mineral fertilizers is possible only when correctly combined with a whole complex of biological preparations and technologies. A large-scale application of biological nitrogen which is most clean ecologically will save us and our children from the danger of chemical pollution of air, water and food, it will decrease the risk of injury to future generations.

Increase in yields during the recent years has been achieved mainly by introduction of crops belonging to intensive varieties which require increased doses of fertilizers and, above all, nitrogenous ones. Therefore a problem of compatibility of microorganism genotypes with a host plant has arisen. The studies of interrelations between microorganisms and host plants are being actively developed in Russia. It is important that the newly created varieties should be able to solve their problems with the help of microorganisms rather than be entirely dependent on the chemical industry.

No obvious role is played so far by the biopreparations despite availability of well developed production processes for a number of highly effective preparations of a new generation. This is explained in the first place by a lack of large production basis which could ensure a wide scale production of biopreparations and their application in agriculture in the required quantities. In Russia the preparations are produced only at pilot enterprises which can produce the amount required for treating up to 1 million hectares whereas the general demand exceeds 20 million hectares. Modern procedures for applying the biopreparations to ensure their wide scale application have not been sufficiently developed. The existing peat form of the preparation is not quite suitable for bulk usage.

The tasks, which the scientists studying biological nitrogen fixation are facing, are urgent. Unfortunately, for an extended period, the research on nitrogen fixation in Russia has been conducted in isolation from similar activities in other countries. It is my firm belief that a close collaboration of scientists and practical workers of Russia with their foreign counterparts will make it possible to develop procedures for ensuring rational agricultural production with maximum utilization of the biological process, thus making agriculture stable, highly effective, ecologically safe and economical.

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THE STRUCTURES OF THE NITROGENASE PROTEINS - AN OVERVIEW

SMITH, B.E., ROE, S.M., YOUSAFZAI, F.K.
Nitrogen Fixation Laboratory, University of Sussex, Brighton,
BN1 9RQ, U.K.

At this Congress there is a half-day formal session on the function of nitrogenases together with a workshop on the same subject. Therefore the object of this overview is to provide an analysis of the structural data upon which much of the interpretation of mechanistic experiments is now based.

Protein crystallographers admit that the resolution of the data obtained for most protein structures is insufficient for them to define the exact positions of atoms. Accordingly they fit `models' to the electron densities obtained: if the intensity is high then perhaps metal or sulfur atoms are indicated; the shape of electron densities associated with specific amino acid side chains is usually characteristic and the bond lengths are known and can be fitted with some accuracy. However, when unknown structures, particularly of metal clusters, are met then there must be an element of guesswork. It is possible by using different X-ray wavelengths to highlight specific atoms and thus, by difference Fourier analysis, to identify their positions with some certainty. Another important source of information is EXAFS spectroscopy which provides accurate atom-to-atom distances, although if there is more than one similar interaction in a cluster only averages can be indicated. A variety of other spectroscopic methods can also provide useful data.

Currently only two groups, Rees from Caltech and Bolin from Purdue, have produced crystallographic data on the nitrogenases; the former has worked largely with *Azotobacter vinelandii* nitrogenase, and the latter largely with *Clostridium pasteurianum* nitrogenase, although each group has worked on the other species. Below we attempt to synthesise the data from both groups to indicate those aspects where there is agreement and those where there still remain some differences.

All molybdenum nitrogenases consist of two metallo-proteins: the iron protein, a γ_2 dimer of equivalent subunits containing a single Fe_4S_4 cluster; and the molybdenum-iron protein, which is an $\alpha_2\beta_2$ tetramer containing two molybdenum and about 30 iron and acid labile sulfur atoms distributed into two types of cluster, the iron-molybdenum cofactor and the P

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cofactor and the P cluster. It is well established that the Fe protein passes electrons to the MoFe protein in a reaction which requires hydrolysis of MgATP to MgADP. Within the MoFe protein the substrates N_2, H^+, C_2H_2, etc. are almost certainly reduced at the FeMoco site. Alternative nitrogenases are also known where molybdenum is replaced by vanadium or possibly just by iron (Eady, 1995). These alternative nitrogenases contain an extra subunit in the larger protein which is apparently essential for N_2 reduction but not other substrate reductions (Waugh et al., 1995).

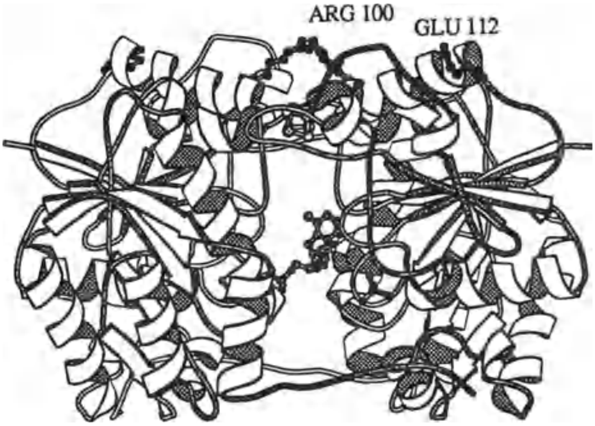


Fig. 1 The structure of the Fe protein from *A. vinelandii*, drawn using MOLSCRIPT and the coordinates of Georgiadis et al., 1992.

The structure of Av2 (the Fe protein from *A. vinelandii*) (Georgiadis et al., 1992) is shown in Fig. 1. The two subunits are related by two-fold symmetry axis which passes through the Fe_4S_4 cluster which is bonded to four cysteine residues, two provided by each subunit. Each subunit consists of a single α/β domain with an eight-stranded β sheet flanked by nine α-helices. In the structure (Fig. 1) an ADP molecule is shown. This was observed as an approximately half occupied site in the Av2 structure but apparently not in the Cp2 structure. Its phosphate end is associated with a previously identified (Robson, 1984) Walker motif A, GXXXXGKS/T, phosphate binding site (Walker et al., 1982) which binds molybdate in the Av2 structure. The ADP spans the two subunits and there is room for two such molecules, with complementary binding sites in each subunit. These are almost certainly the two MgATP or MgADP sites identified in binding experiments with the Fe proteins from a number of species (Howard, Rees, 1994). Recent site-directed mutagenesis experiments which probe these sites are fully consistent with this view (Seefeldt, Mortenson, 1993).

However this nucleotide binding site is about 20 Å from the Fe_4S_4 cluster although binding of nucleotides modifies the redox potential, EPR signal and access to chelators of the iron-sulfur site. These observations indicate that conformational changes occurring upon nucleotide binding must be allosterically transmitted to the iron-sulfur site. It has been suggested (Howard, Rees, 1994) that this transmission is through changes in salt bridges between the subunits as the nucleotides bind.

There is mounting evidence (Howard, Rees, 1994) that the surface which includes the 'top' of the molecule above the Fe_4S_4 cluster is the surface that interacts with the MoFe protein. In photosynthetic species Arg100 is ADP ribosylated (Murrell et al., 1988) in a regulatory reaction thus preventing interaction with the MoFe protein: cross-linking experiments between Av2 and Av1 identified Glu112 as close to the MoFe protein (Willing et al., 1989). Most recently, Peters et al. (1994) replaced residues 59 to 67 in Av2 with comparable residues from Cp2. These residues also form part of the 'top' surface. The rationale behind this experiment was that the Cp2 and Av1 form a tight inactive complex, therefore this replacement might affect the interaction between the Fe and MoFe proteins. In support of this hypothesis the activity of the hybrid protein was reduced by a factor of two and this loss of activity was associated with a much slower dissociation rate between the hybrid Av2 and Av1.

The molybdenum-iron proteins (Av1 and Cp1)

Bolin et al. (1990) showed that the FeMoco sites were 70 Å apart; that the P clusters were likely to be Fe_8 clusters, rather than the two Fe_4 clusters as expected from spectroscopic evidence, and that the FeMoco and P clusters were separated by about 19 Å. The full crystallographic structures of both Av1 (Kim, Rees, 1992b) and Cp1 (Bolin et al., 1993; Kim et al., 1993) proteins show that the α and β subunits have similar folds, each consisting of three α/β domains. The FeMoco clusters are found completely within the α subunits, whereas the P clusters bridge the α/β interfaces. The Av1 structure is shown in Fig. 2 and can be considered as a pair of interacting α/β dimers. There are extensive contacts between the α and β subunits in each dimer and the tetramer is produced by extensive α-helical interactions between the β subunits with some additional α-β interaction. The FeMoco centres are at least 10 Å below the surface in a largely hydrophilic environment, whereas the P clusters are a similar distance below the surface in a largely hydrophobic environment. An unusual open-end channel about 10 Å in diameter can be seen in the tetramer. The tetramer interface is also stabilised by a cation binding site with ligands from both β subunits and probably containing magnesium or calcium ions.

The FeMoco clusters are bound to the protein by only two ligands, His442 and Cys275 (Av1 residue numbers). Homocitrate is bound to the molybdenum atom by its 2-hydroxy and 2-carboxy groups and extends from the FeMoco centre towards the P clusters. The homocitrate end of FeMoco is surrounded by at least 12 water molecules (Bolin et al., 1993). His195 is not a ligand to the cofactor but is almost certainly hydrogen bonded to one of the sulfur atoms (Kim et al., 1995). Site-directed modification of this residue results in changes in spectral and activity properties.

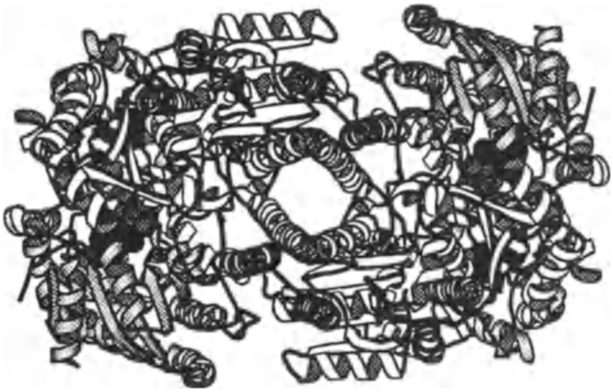


Fig. 2 The structure of the MoFe protein of *A. vinelandii* drawn by MOLSCRIPT from the coordinates of Kim, Rees, 1992b. The α subunit chains are shaded and β subunits white. The FeMoco and P clusters are represented by space-filling models.

FeMoco (Fig. 3) can be considered as being derived from $MoFe_3S_4$ and Fe_4S_4 clusters, each of which has lost a sulfur atom and which are then bridged by three additional sulfur atoms between iron atoms. Earlier work (Kim, Rees, 1992a) had indicated that one of these sulfur atoms might be an atom of lower atomic number, *e.g.* N or O. However evidence from Cp1 (Bolin et al., 1993) indicates that all the bridging atoms have similar electron densities which are within experimental error of the electron densities of other sulfur atoms in the protein. An earlier suggestion that there might be a hexavalent sulfur atom at the centre of the cluster bonded to each iron atom has now been ruled out, although it is not yet possible unequivocally to rule out a lower atomic number atom in this site (J. Bolin, personal communication). The metal-metal and metal-sulfur distances in the current model fit well with those derived from EXAFS spectroscopy (J. Bolin, personal communication). However there are six unusual trigonal iron atoms in the FeMoco. Iron normally has at least four ligands in metal complexes. If each of these iron atoms is considered in relation to the plane formed by its three bonded sulfur atoms it is displaced from that plane towards the centre of the molecule. This could imply that there is some iron-iron bonding between the apparently trigonal iron atoms in the FeMoco centre (J. Bolin, personal communication).

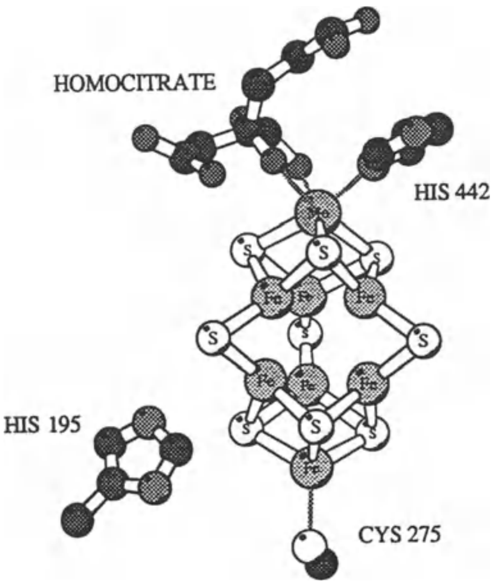
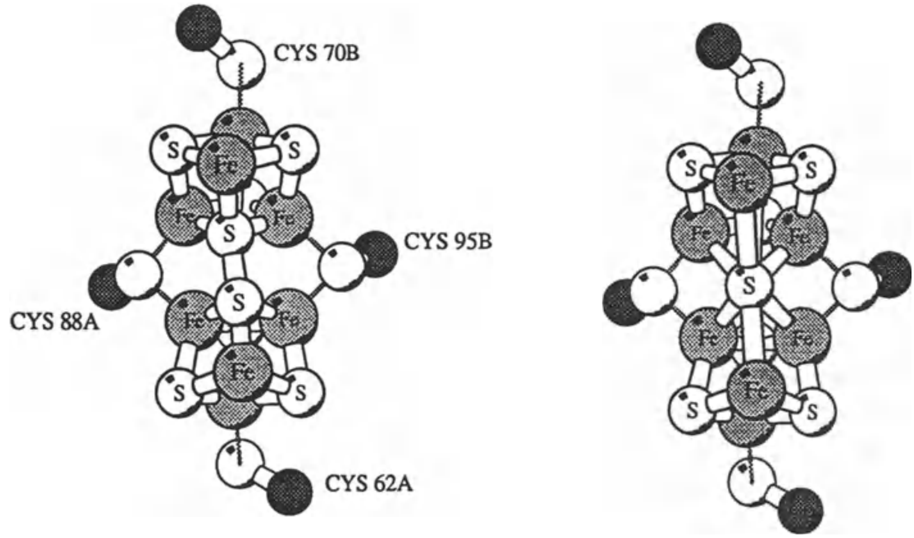


Fig. 3 The iron-molybdenum cofactor of nitrogenase.

The P clusters are bonded at the interface of the α and β subunits with cysteine ligands with three cysteines provided from each subunit and two bridging cysteine residues. Originally (Kim, Rees, 1992a) the P clusters were thought to be simply two Fe_4S_4 clusters bridged in this manner. Later work (Bolin et al., 1993) indicated that two of the corner sulfur atoms were also closely interacting (Fig. 4A). The extent of this interaction is still a matter of dispute. Rees' group indicates (Kim et al., 1993) that there is a sulfur-sulfur bond of 2.1 Å, although older crystals appear to have lost one of these sulfur atoms. Bolin's data are more consistent with an Fe_8S_7 structure (see Fig. 4B) (J. Bolin, personal communication). Attempts to fit two sulfur atoms lead to distortion of the geometry of the clusters and unusual B factors for the sulfur atoms which are inconsistent with those derived for the other atoms in the clusters. A further difference between the two groups is that in Av1 Serβ188 apparently bonds one of the iron atoms, whereas in Cp1 the equivalent residue hydrogen bonds to a sulfur atom.

Thus there are still difficulties concerning the nature of the P clusters. It is essential that these differences are resolved since it is clear that nitrogenases contain two types of metal clusters which are unique in biology and are presumably both somehow essential for the difficult chemistry that the enzyme has to carry out. The function of both clusters must be important although substrates almost certainly bind to the FeMoco centre.

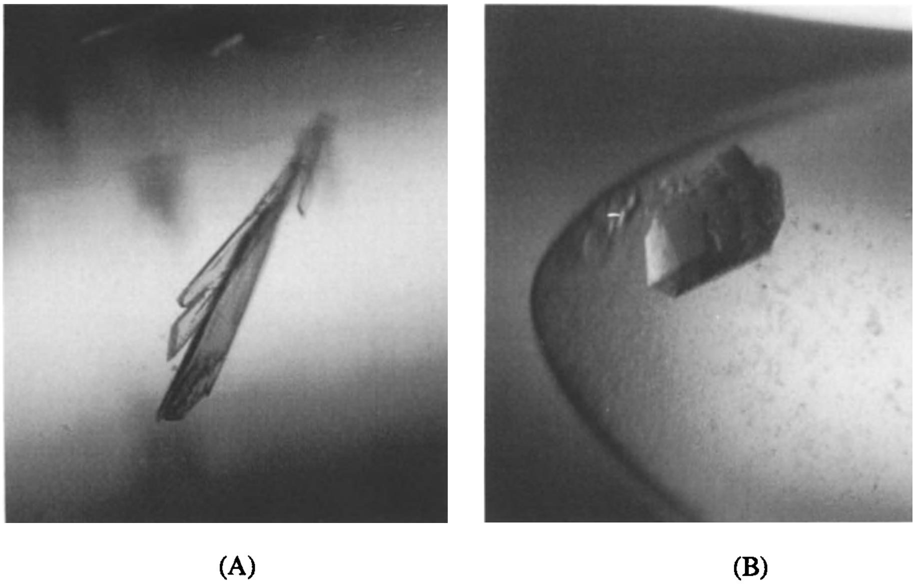


(A) (B)

Fig. 4 Fe_8S_8 (A) and Fe_8S_7 (B) fits to the electron density associated with the P clusters. Cysteine residues α154 and β153 have been removed for clarity.

Recent work in this Laboratory with extracts from a double mutant in which both of the bridging cysteine residues to the P clusters have been replaced by alanine has shown that three forms of active Kp1 can be isolated. These three forms can be separated on FPLC Q sepharose indicating that they have different surface charges and therefore presumably are associated with different folds of the $\alpha_2\beta_2$ tetramer. The activities of each of the forms is lower than that of the wild-type but the ratio of electrons donated to various substrates is unchanged as is the ATP/2e ratio. Each of the species exhibits characteristic 4.3, 3.7, 2.1 EPR signals associated with the FeMoco centres. The intensity of this EPR signal correlates with the activity of the species. From this evidence we conclude that in those molecules which contain FeMoco the P clusters are fully competent despite the mutations carried out, *i.e.* these mutations replacing the bridging cysteine ligands do not affect the rate-limiting step in nitrogenase turnover. Further work is required to analyse the partial reactions of the enzyme in order to determine which steps in its turnover are affected by these mutations.

It is clear from the above that additional structural information is needed on the MoFe proteins before definitive structures can be agreed. One approach which has been successful in many other instances is to analyse the crystal structure of an alternative species. In this Laboratory we have had some success with obtaining crystallographic data on the MoFe protein from *Klebsiella pneumoniae*, Kp1. Two forms of crystals have been obtained, those shown in Fig. 5A are thin monoclinic plates usually associated with some precipitation of the protein. A full data set of 3 Å has been collected on these crystals and is being refined. A second crystal form (Fig. 5B) has recently been obtained which diffracts to at least 2.6 Å.



(A) (B)

Fig. 5 Crystal forms of the MoFe protein from *Klebsiella pneumoniae.*

Interactions between the Fe protein and the MoFe protein

Fig. 6 shows proposed (Kim, Rees, 1992b) interaction sites between Av2 and half a molecule of Av1. As indicated above in Fig. 1, the 'top' surface of Av2 including the iron-sulfur cluster is thought to contact Av1. The evidence for the interacting surface of Av1 is less well established but includes the cross-linked lysine observed by Willing et al. (1989) and evidence that phenyl alanine 125 modification affects the dissociation rate of the two proteins (Thorneley et al., 1993). Aligning the two proteins in the manner shown locates the Fe_4S_4 cluster of Av2 14 Å from the P cluster which in turn is about 14 Å from the FeMoco centre (edge-to-edge). These geometric relationships have been interpreted in terms of electrons passing from the iron protein through the P clusters to FeMoco but it should be stressed there is as yet no evidence that all electrons to FeMoco take this route.

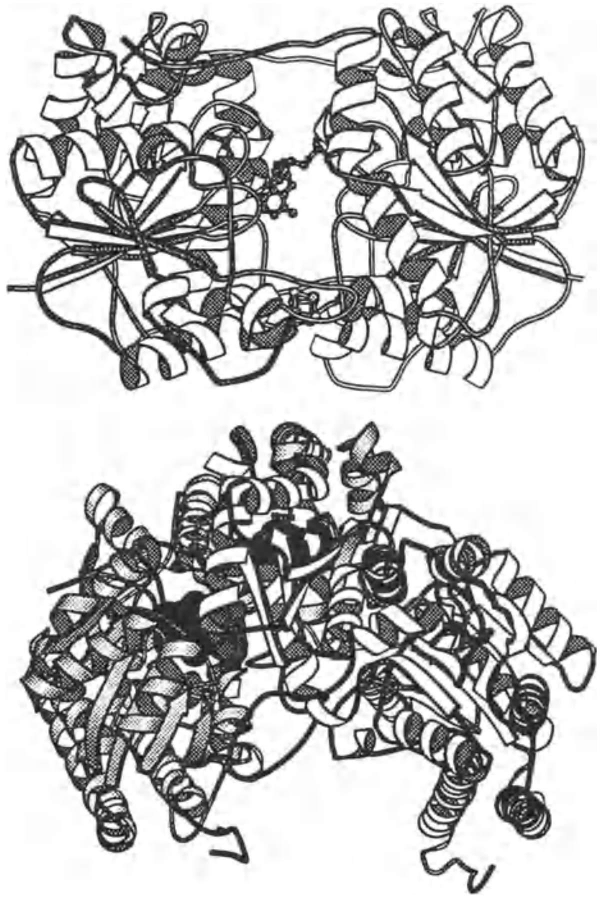


Fig. 6 Possible interaction mode between the Fe protein (upper) and MoFe protein (½ molecule only shown, lower) after Kim, Rees, 1992b.

Conclusions

Although there is now a reasonable consensus on the structure of the FeMoco centre the structure of the P clusters is still contentious. Nevertheless the available structures are of major importance and form a reasonable basis on which to build testable hypotheses on the mechanism of nitrogenase.

Acknowledgements

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MOLECULAR GENETICS OF NITROGEN FIXATION IN PHOTOSYNTHETIC PROKARYOTES

HASELKORN R.
Department of Molecular Genetics & Cell Biology, The University of Chicago, 920 East 58 Street, Chicago IL 60637 USA

Nitrogen fixation in photosynthetic bacteria and in cyanobacteria is characterizd by several features of *nif* gene organization and regulation not encountered in the well-studied diazotrophs *Klebsiella pneumoniae* and *Azotobacter* species. The filamentous cyanobacteria, in addition, differentiate specialized cells for nitrogen fixation, the heterocysts, and this cellular process requires the coordinated expression of many genes, up to one-fifth of the 7-Mb genome of *Anabaena* species. Significant progress has been made, in recent years, in the elucidation of these novel features in two model organisms, the purple non-sulfur bacterium *Rhodobacter capsulatus* and the cyanobacterium *Anabaena*. In order to appreciate these novelties, it will be useful to recall the essential elements of *nif* gene organization and regulation in *Klebsiella* and *Azotobacter*. The key regulatory genes are *ntrB, ntrC, nifA* and *nifL*. All of the genes encoding nitrogenase components, FeMoCo synthesis factors, assembly factors and electron transfer proteins are transcribed from promoters recognized by σ54-RNA polymerase, a form of holoenzyme that requires activation by the enhancer-binding proteins NtrC or NifA. In the case of most *nif* genes, the enhancer binds NifA. The exception is *nifA* itself, whose enhancer binds NtrC. In these bacteria, oxygen represses *nif* gene expression (indirectly) by oxidizing the redox protein NifL which, in oxidized form, prevents the binding of NifA to its enhancer sequences. Ammonia represses at two levels: by converting NifL to a NifA-binding form and by preventing transcription of the *nifA* genethrough the action of NtrB and NtrC. Details of the role of NifL are described in the article by R. A. Dixon elsewhere in this volume. NtrB and NtrC are an environment-sensing pair, in which NtrB is the sensor. When the level of fixed nitrogen falls, it phosphorylates NtrC. The phosphorylated form of NtrC binds to its enhancer sequences and its ATPase activates σ54-RNA polymerase.

The regulatory system in *Rhodobacter capsulatus* differs from the one just described in several ways. First, the only σ54 equivalent discovered in *Rhodobacter* thus far, encoded by the *nifR4* gene, appears to be requied only for transciption of the *nif* structural genes and perhaps a few others. In particular, it is not required for amino acid transport and utilization or for transcription of the *nifA* gene (of which there are two). On the other hand, both *nifA* genes require the NtrB/NtrC pair, encoded by the *nifR1*

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and *nifR2* genes, to be expressed. The laboratory of R. G. Kranz has carried out a detailed mutational analysis of NtrC of *R. capsulatus* and defined its DNA-binding sequences. These studies show clearly that *nifA* transcription requires the upstream binding of the active form of NtrC, but they leave open the question of which form of RNA polymerase does the job (Foster-Hartnett et al, 1994). W. Klipp, elsewhere in this volume, describes genes for molybdate transport whose transcription requires NtrC but not σ54. Thus it seems likely that another σ factor is needed for transcription of *nifA*. Both Kranz and Klipp have shown that the *nifR4* gene, which encodes σ54, is regulated in part by NifA! The article by Klipp contains further descriptions of the regulatory circuits controlling expression of genes for the alternative nitrogenase in *R. capsulatus*, including evidence for yet another σ factor and a role for a gene from the photosynthesis gene cluster, *hvr*, in ammonia regulation of *nif* gene expression.

Several laboratories, my own included, have screened many thousands of colonies in search of a mutation in one of the genes encoding additional σ factors required for *nif* gene transcription. All of these searches have failed, possibly because the σ factors are responsible for transcription of essential genes as well. For that reason, Su Zheng in my laboratory cloned the gene encoding the principal σ factor of *R. capsulatus* and sequenced it. The protein, whose size puts it closer to the σ factors of *Bacilli* and cyanobacteria than to the σ70 of *E. coli*, is similar in sequence to these factors, particularly in the regions beleieved to interact with the -10 and -35 hexamers that comprise the consensus *E. coli* promoter. Two apparent transciption start sites for this *sigA* gene were identified by primer extension, but the one further from the coding region was not confirmed by *lacZ* fusions. The single remaining start site is about twice as active in nitrogen-derepressing conditions as in complete medium, whether measured by primer extension or *lacZ* fusion activity (S. Zheng, to be published).

The model proposed by Klipp (this volume) for the interplay of regulatory factors controlling the two nif systems contains multiple feedback loops. The model explains beautifully how the wild-type organism responds to various environmental conditions and the physiological consequences of some mutations. The loops are so extensive and ramified that it is difficult to see all of the consequences, however. For that reason, and others, we have turned to a global approach for studying the *R. capsulatus* chromosome. We have assembled an ordered set of 200 cosmids, selected from a library of more than 1700, that gives complete coverage of the 3.7-Mb chromosome. More than 3600 restriction sites for four enzymes have been mapped for the type strain SB1003 (Fonstein et al, 1995). All of the genes of *R. capsulatus* reported by the end of 1994 have been mapped by hybridization to digests of the cosmid set. These digests, displayed in gene or operon-sized fragments in 200 lanes of a master gel, provide a template to be used for analysis of transcription under various conditions and in various mutant strains.

Cyanobacteria fix nitrogen in both the free-living and symbiotic state. The principal paradox these organisms present to investigators is their ability to do this while generating oxygen through photosystem II. Some cyanobacteria, both unicellular and filamentous, protect their nitrogenase by vigorous respiration, which produces the ATP needed for fixation and removes oxygen at the same time. Other cyanobacteria, such as *Anabaena*, differentiate specialized cells for nitrogen fixation, called heterocysts, at regular intervals along the filaments. These cells have an internal reducing environment achieved by shutting off photosystem II and reducing diffusion of oxygen into the cell by synthesizing a double-layered envelope of polysaccharide and glycolipid. A culture of wild-type *Anabaena* 7120 growing on a medium with ammonia as nitrogen source shows no sign of cellular differentiation. When such a culture is transferrred to a medium lacking ammonia, about 10% of the cells begin to differentiate into heterocysts. The developing cells are located at the ends of the filamants and at approximately equal intervals within each filament. (See Figure 1.) This pattern is maintained by the differentiation of the vegetative cell placed midway between each heterocyst, with the time required for differentiation about equal to the time required for each vegetative cell to divide once. For *Anabaena* 7120, mature heterocysts begin to appear 15-18 hours after nitrogen step-down at room temperature under aerobic conditions and that is the time at which nitrogenase activity is first detected. Similar kinetics of nitrogenase appearance are seen under microaerobic conditions. The strain called *Anabaena variabilis* 29413, as shown by T. Theil and by B. Schrautemeier et al at this Congress, contains a second set of genes encoding a Mo nitrogenase that are expressed under microaerobic conditions in vegetative cells within the first few hours after nitrogen step-down. H. Bothe describes evidence for additional forms of nitrogenase in this strain elsewhere in this volume.

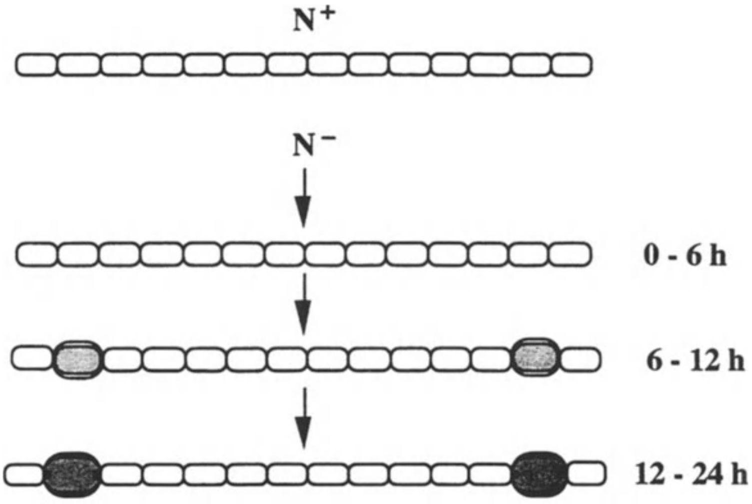

Figure 1. Heterocyst differentiation in wild-type *Anabaena* 7120.

Anabaena 7120 does not appear to have more than the single set of Mo nitrogenase genes. In *Anabaena* 7120, two of the *nif* operons and a third operon encoding hydrogenase contain interrupting DNA elements. The elements interrupting the *nif* genes must be removed in developing heterocysts, by site-specific recombinases, in order to get full-length transcripts of the *nif* operons. Without these rearrangements, there can be no active nitrogenase. Expression of the genes encoding the recombinases is normally a late event in heterocyst differentiation (Haselkorn, 1992). Mutation of these genes results in a Nif-phenotype; the cells cannot grow on N_2 as nitrogen source. Mutation of any of the genes required early in heterocyst differentiation also results in a Nif-phenotype. On the other hand, mutation in a gene required for heterocyst glycolipid synthesis or assembly results in a heterocyst that cannot provide adequate protection from oxygen. Such mutant strains are said to have a Fox-phenotype: they can fix nitrogen and reduce acetylene under microaerobic conditions, but not in air. This ability to fix microaerobically implies that the *nif* gene rearrangements can occur in oxygen-sensitive cells.

During the past few years, C. P. Wolk and colleagues have used Tn*5* carrying the *lux* genes of *Vibrio* to construct chromosomal fusions in *Anabaena* 7120, then used the luciferase activity of the fusion strains to identify genes whose transcription is activated during heterocyst development. In some cases, it was possible to identify mutant phenotypes due to the Tn*5* insertion and then to use the Tn*5* selection in *E. coli* to clone the affected gene. One important regulatory gene identified this way, *hetN*, will be described below (Black, Wolk, 1994). We have used chemical mutagenesis instead to induce mutations in *Anabaena* 7120, then applied several rounds of penicillin selection to collect a set of Nif mutant strains (Buikema, Haselkorn, 1991a). These could be classified as early acting, such that heterocyst differentiation did not occur or the spacing pattern was altered, or late acting, in which the heterocysts that differentiated did not provide adequate prrotection from oxygen or that were easily detached from neighboring vegetative cells. The phenotypes of strains mutated in each of three early acting genes are shown in Figure 2.

The earliest acting of these genes is *hetR* (Buikema, Haselkorn, 1991b). Transcripts of this gene are seen in cells growing on ammonia, at a low level. Within an hour of nitrogen step-down, increased abundance of *hetR* transcripts can be seen. A *hetR* mutant strain, either point mutation or null mutation, fails to initiate heterocyst differentiation. The only sign that a nitrogen starvation signal is perceived is the proteolysis of the phycobilproteins. The wild-type *hetR* gene was isolated by complementation of the HetR mutant strain, using a libary of cosmids containing wild-type DNA fragments in a conjugable vector. The HetR sequence does not betray the precise function of the protein. It is, however, a master regulatory gene as seen from the following experiments. First, when the wild-type gene is placed on a plasmid into the wild-type strain, the strain makes too many heterocysts, even in medium containing nitrate or ammonia (Figure 2). Second, Buikema has constructed a vector in which any cloned gene can be expressed from the promoter of the *petE* gene of *Synechococcus*, the kind gift of Neil Straus. This promoter is tightly regulated by Cu ion concentration in the medium, shown by Straus for *Synechococcus* and confirmed by Buikema for *Anabaena* 7120. Buikema then used this vector to drive transcription of the *hetR* gene in *Anabaena* 7120, varying the level of HetR by varying the level of Cu ions. When the *hetR* gene was turned on, complete heterocyst differentiation could be observed, even in medium containing ammonia (W. J. Buikema, to be published).

A second early-acting gene is *patA*, mutation in which results in a strain that differentiates heterocysts only at the ends of filaments (Liang et al, 1992). A *patA* mutation suppresses the multi-heterocyst phenotype due to extra copies of the *hetR* gene (Figure 2). The PatA sequence suggests that it is the regulator of a two-component system, perhaps analogous to NtrC. This information led us to look for histidine kinase genes, in the hope of finding one that is paired with *patA*. Michael Fath and Eliz Koshy undertook this search in my lab, using the PCR and a set of primers corresponding to

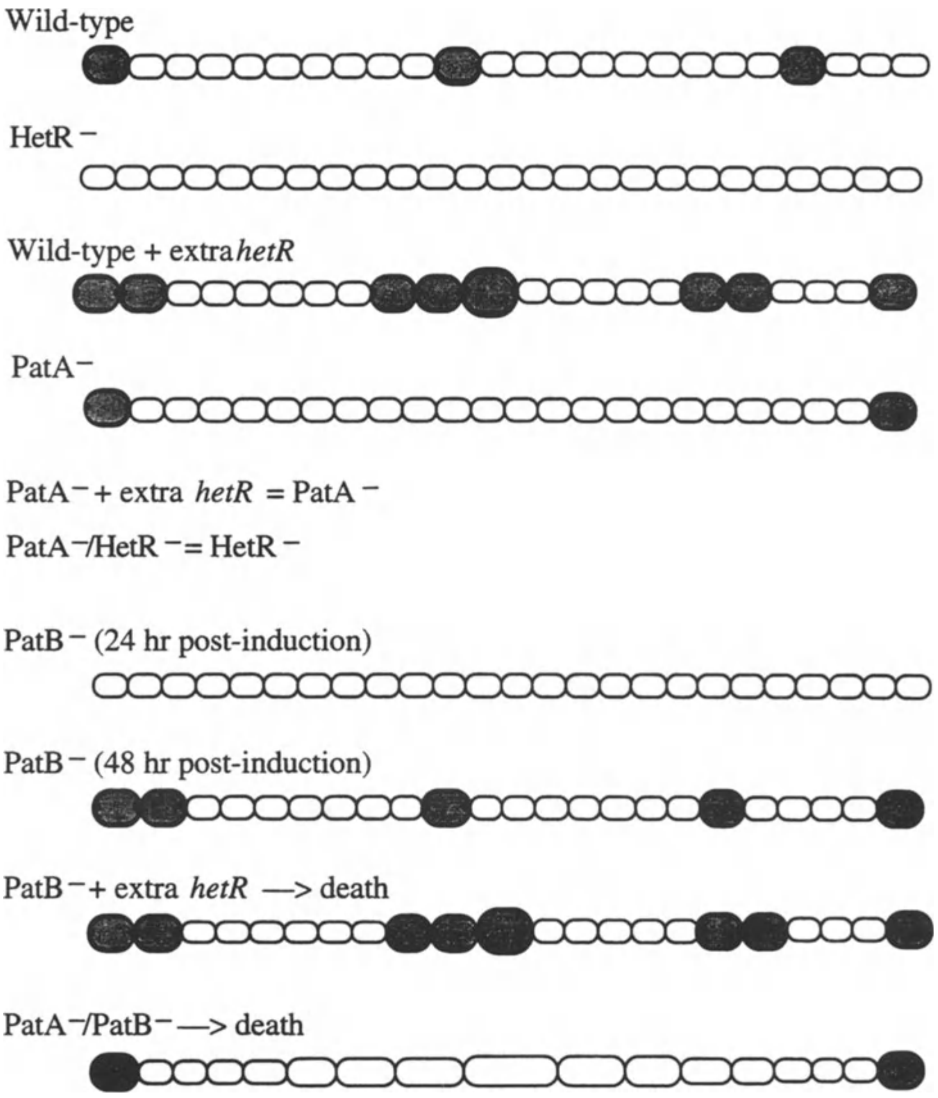
Wild-type
HetR$^-$
Wild-type + extra *hetR*
PatA$^-$
PatA$^-$ + extra *hetR* = PatA$^-$
PatA$^-$/HetR$^-$ = HetR$^-$
PatB$^-$ (24 hr post-induction)
PatB$^-$ (48 hr post-induction)
PatB$^-$ + extra *hetR* —> death
PatA$^-$/PatB$^-$ —> death

Figure 2. Appearance of *Anabaena* 7120 mutant strains on N-free medium.

three families of bacterial histidine kinases in all combinations of forward and reverse primer pairs. This approach yielded seven candidates, called *askA* to *askG*, all of which have been used as probes to find the complete genes in a cosmid library. To date, one of the corresponding genes has been inactivated in *Anabaena* 7120, with no consequence for the developmental phenotype. A second gene, when inactivated, results in a more random spacing of heterocysts than seen in the wild type (M. Fath et al, to be published). A third mutation, in the *patB* gene, results in a phenotype that is difficult to characterize because it is not the same in liquid culture and on plates (Liang et al, 1993). On nitrogen-free plates, colonies of the *patB* mutant strain bleach around the edges and the colonies grow very slowly. In liquid, bleaching is less evident, heterocyst development is delayed by a day with respect to wild type (Figure 2) and eventually the heterocyst frequency is higher. Acetylene reduction activity is the same as wild type on a chlorophyll basis, but the vegetative cells grow very slowly. If the gas phase is supplemented with CO_2 up to 2%, then the timing of heterocyst development approaches that of the wild type, but growth is still very slow. Mutation in *patB* does not suppress the development of extra heterocysts in the multi-copy *hetR* strain. A strain in which both *patA* and *patB* are mutated dies rapidly following nitrogen step-down, creating large empty cells in the process (Figure 2). The PatB protein sequence suggests that it is a redox-sensitive DNA-binding protein: it has a ferredoxin domain at the N-terminus and a helix-turn-helix structure near the C-terminus (Liang et al, 1993). None of the three genes just described is needed for growth on ammonia. All three mutations are recessive to the corresponding wild-type allele.

One more early-acting gene has been characterized in two labs independently. E. Flores and colleagues found a gene in *Synechococcus* required for transcription of many genes for nitrogen assimilation and named it *ntcA*. They later cloned the homologue from *Anabaena* 7120 and found that it was required early for heterocyst differentiation. At the same time, J. W. Golden and colleagues cloned a gene from *Anabaena* 7120 encoding a DNA-binding protein, shown earlier to bind to a sequence upstream from the *xisA* gene, which encodes a site-specific recombinase required to rearrange the *nifHDK* operon. The gene for this binding factor turns out to be *ntcA*. Now NtcA binds to a consensus sequence and NtcA is needed for the transcription of the *hetR* gene, but the *hetR* gene does not have the NtcA binding site near any of its multiple promoters (W. J. Buikema, unpublished). Therefore, the mechanism by which NtcA activates *hetR* must be indirect, possibly involving elements of two-component cascades. NtcA transcription is more or less constitutive (J. W. Golden, T. Ramasubramanian, posters), so its perception of nitrogen status ought to be reflected in some form of modification, but that remains to be determined.

The search for mutations that affect heterocyst structure has turned up several interesting classes. The Wolk lab found one mutation in a gene that is required for assembly or transport of the heterocyst polysaccharide layer. This strain cannot provide oxygen protection for nitrogenase. We have several mutants with the same phenotype, but different genes are affected. In one case, the mutation affects a gene called *hglK*, which encodes a protein with four putative membrane spans followed by 36 repeats of the pentapeptide sequence AXLXX (K. Black et al, to be published). This strain lacks the glycolipid layer although it has wild-type levels of the heterocyst-specific glycolipids (C26 and C26 fatty acids in a-glycosidic linkage to glucose) in the cell interior. For that reason, we believe that the HglK protein transports the glycolipids. Another set of mutants, in genes called *hglB, hglC* and *hglD*, fail to make the glycolipids at all. The sequences of these genes suggest that they encode enzymes for the synthesis of the fatty acid chains of the heterocyst-specific glycolipids. There is a complicated matter associated with this fatty acid gene cluster. Within the cluster is the ***hetN*** gene referred to previously. When this gene is placed on a plasmid and introduced into the wild type, it prevents heterocyst differentiation. Mutation of this gene was originally thought to prevent differentiation also, but most likely the original mutation derepressed differentiation, a toxic condition for cells growing on nitrate or ammonia, and they acquired secondary mutations in the differentiation pathway. Our current opinion is that HetN is involved in the synthesis of a negative regulator of differentiation in vegetative cells.

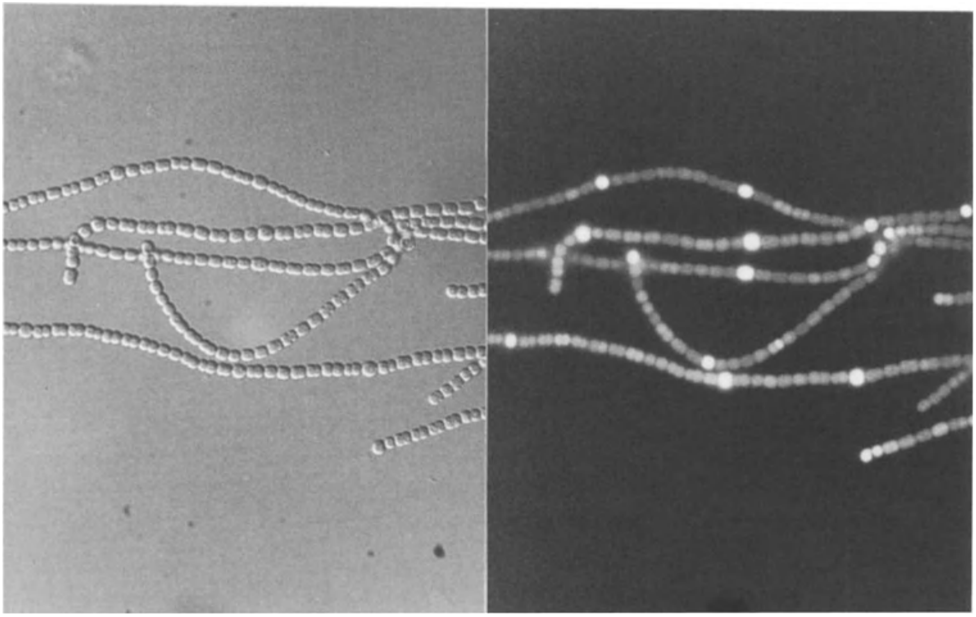

Figure 3. Expression of the *hetR* gene in wild-type *Anabaena* 7120 revealed by fluorescence from the GFP reporter. Nomarski image on the left shows heterocyst locations; fluorescence on the right reports the gene expression.

Returning to the early acting genes, I want to describe some unpublished work of W. J. Buikema that promises to provide answers to the questions of the relationships among the regulatory genes described to date and to provide a route to the discovery of other genes in the circuit. His experiments involve a new reporter for gene expression that is convenient to use, does not require very expensive equipment, can be monitored continuously, and is cell-autonomous. This reporter is the Green Fluorescent Protein (GFP) gene from the jellyfish. When this protein is expressed in *Anabaena*, it can be seen clearly, with low backgound from chlorophyll, by exciting the fluorescence in the blue and measuring the emission in the green. A vector was constructed with a promoterless *gfp* gene followed by a *cat* gene, and a cloning site preceded by transcription terminators, all in a plasmid that can be conjugated into *Anabaena* 7120 and will replicate there. The promoter region of the *hetR* gene was inserted in the cloning site, creating a *hetR::gfp* transcriptional fusion. This plasmid was conjugated into the wild type and several mutants of *Anabaena* 7120. Figure 3 shows the fluorescence due to synthesis of GFP driven by transcription of the *hetR* promoter in wild type filaments 25 hours after nitrogen step-down. The Nomarski image is shown for comparison. First, it is clear that *hetR* is transcribed abundantly in differentiating cells but not in vegetative cells. A second point is that the fluorescence decreases in older heterocysts. We believe that the latter effect is due to the strong reducing environment in heterocysts. It is known that the fluorescence of GFP requires that the protein be oxidized. When the same reporter plasmid was conjugated into the HglK mutant, which cannot provide protection of nitrogenase from oxygen, the heterocysts remained bright. Introduction of the reportr plasmid into the PatA mutant strain resulted in bright cells mainly at the ends of filaments, that is, only where the heterocysts developed. Finally, when the plasmid was put into the HetR mutant strain, the level of fluorescence increased in all the cells following nitrogen step-down and it stayed high in many of them. This result suggests that HetR is autoregulatory in two ways: positive in heterocysts and negative in vegetative cells.

In summary, there is almost enough information available to start constructing models for the regulatory circuits involved in heterocyst differentiation and nitrogen fixation in *Anabaena*, but much of the information on hand for organisms such as *R. capsulatus*, *Azospirillum*, and *Azorhizobium*, to say nothing of the well-characterized *Klebsiella*, *Azotobacter* and *Rhizobium* is still missing for *Anabaena*. We do not have NtrB/NtrC homologues, there is no s54, there is no NifA. We do not know how the nitrogen signal is read by NtcA, how it is transmitted to HetR, or how HetR turns on the complete process of differentiation. The roles of PatA, PatB, HetN and all the kinase genes remains to be determined. We have high hopes for the GFP reporter to help sort out these relationships.

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SIGNALS INVOLVED IN NODULATION AND NITROGEN FIXATION

LUGTENBERG, B.J.J. (1), BLOEMBERG, G.V. (1),
VAN BRUSSEL, A.A.N. (1), KIJNE, J.W. (1), THOMAS-OATES J.E.(2)
AND SPAINK, H.P. (1).
(1) Institute of Molecular Plant Sciences, Leiden University,
Wassenaarseweg 64, P.O.Box 9505, 2300 RA Leiden,
The Netherlands.
(2) Utrecht University, Dept. of Mass Spectrometry, Sorbonnelaan 16,

INTRODUCTION

One of the most fundamental aspects in biology is the regulation of biochemical pathways, which is brought about by an interplay between signals, genetic elements and transacting factors. Our knowledge of the signals involved in nodulation and nitrogen fixation is progressing rapidly but our understanding of their mechanisms of action is rather poor. In this overview we will deal with signals known or assumed to be involved in the various steps which lead to nodulation and nitrogen fixation. *Rhizobium* will be the leading microsymbiont but, when appropriate, other microbes will be dealt with. Because of severe space limitation many valuable references had to be omitted.

THE BACTERIUM'S STRUGGLE BEFORE REACHING THE INSIDE OF THE PLANT

When we want to understand plant-microbe interactions we should realize that the physiological conditions under which plant and bacterium interact in nature differ greatly from laboratory conditions. It seems very likely that bacterium and plant often live under conditions of nutrient starvation but low pH, osmotic stress and temperature stress are common as well. Infectivity of *Rhizobium* even appears to depend on the nature of the limitation (Kijne et al., 1988). Gene expression under natural conditions will differ from that under laboratory conditions. Work presently carried out on the adaptation of rhizobia to low pH (Glenn and Dilworth, 1994) and on adaptation to nutrient limitations and temperature stresses (in Frans de Bruyn's laboratory) shows that many genes are involved in the adaptation of the bacterium's physiology to these stress conditions. The future will bring information about the second messengers which play a role in these processes. When applying rhizobial inoculants, one should take these factors into account and also the fact

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that inoculant bacteria have to protect themselves against predators and toxins. Furthermore, legumes such as pea can also establish a symbiosis with several arbuscular mycorrhizal soil fungi belonging to the Glomales (Perotto et al., 1994). The most significant environmental variable controlling the competitive success of rhizobial inoculants is the size of the indigenous rhizobial population (e.g. Thies et al., 1992). Processes which probably play a role in the outcome of competition, and in which signals are involved, include chemotaxis, intercellular communication, toxin production (e.g. trifoli-toxin; Triplett, 1990), polysaccharide synthesis and *nod* gene induction leading to production of lipochitin oligosaccharides (LCOs). The signals expected or known to be involved in these processes will be discussed below.

Chemotaxis

Root exudates contain amino acids, sugars and organic acids as the major components. Their composition can vary with the host genotype. Pea exudate is particularly rich in homoserine (Rovira, 1956). Non-motile and non-chemotactic mutants lose competition with the parental strain for nodulation (Hunter and Fahring, 1980; Ames and Bergman, 1981; El-Haloui et al., 1986; Mellor et al., 1987; Catlow et al., 1990), presumably because chemotaxis towards exudate components is important for competition and survival. Rhizobia appear to be excellent scavengers of organic compounds, and may depend largely on materials that escape utilization by faster-growing bacteria. For example, some rhizobia grow faster on 10^{-7} M succinate than certain strains of *Enterobacter* and *Pseudomonas*, although the latter strains grow faster at higher concentrations (Humbeck et al., 1995).

The topic of chemotaxis to root exudates has recently been reviewed by Zhulin and Taylor (1995). Plant-associated bacteria respond chemotactically to certain compounds released by plants, which allows them to occupy specific ecological niches. Root exudates of leguminous plants attract rhizobia. Rhizobia are strongly attracted to a few sites on the root surface (Gulash et al., 1984; Caetano-Anolles et al., 1992), presumably because these are the sites of metabolite secretion. Among the metabolites secreted in reasonable amounts by plant roots, organic acids are the best attractants for rhizobia. Amino acids and sugars in micromolar concentrations also act as attractants for *Rhizobium* but usually not for *Bradyrhizobium* (Barbour et al., 1991). The spectrum of attractants may vary, not just at the species level but also from strain to strain (Zhulin and Taylor, 1995). The mechanism of chemotactic response in rhizobia is still unknown. In contrast to *Escherichia coli*, no dedicated receptors have been found. Some phenolic compounds can serve as chemoattractants at very low concentrations. The *nod* gene inducers 4^1,7-dihydroxyflavone, 4^1,7-dihydroxyflavone and 4,4^1-dihydroxy-2-methoxychalcone, secreted by alfalfa roots, cause chemotactic responses in concentrations as low as 10^{-10} M, which are independent of *nod* gene induction (Dharmatilake and Bauer, 1992). However, inhibitors of *nod* gene induction like umbelliferone and acetosyringone (Aguilar et al., 1988) and kaempferol (Armitage et al., 1988) can also produce strong positive chemotactic responses in *R.leguminosarum*, although at much higher concentrations. So, in rhizobia induction of chemotaxis is not always linked to *nod* gene expression. In conclusion, rhizobia show positive chemotaxis towards two classes of chemicals secreted by plant roots. Firstly, these bacteria are attracted by nutrients of which the best attractants are organic acids such as succinate and some aromatic acids. Secondly, rhizobial cells show chemotaxis towards flavonoids and some other phenolic compounds which do not serve as nutrients but play an important biological role (Zhulin and Taylor, 1995).

Bacteria of the genus *Azospirillum* are capable of chemotaxis towards almost all common metabolites typical of root exudates such as organic acids, amino acids and sugars (Zhulin et al., 1988) at an optimal concentration of 10^{-3} M. The best growth substrates such as malate, succinate, gluconate and fructose are strong attractants whereas poor substrates, such as amino acids, are weak attractants. Recently it was demonstrated that *Azospirillum* has a highly sensitive chemotactic response towards aromatic acids that are characteristic of the rhizosphere, such as benzoate, protocatechuate and hydroxy-benzoate. Treshold levels range from 10^{-6} to 10^{-11} M (Lopez de Victoria and Lovell, 1993).

Recent work in Jos Vanderleyden's laboratory has shown that *Azospirillum* can make two types of flagella. Firstly a constitutively synthesized polar flagellum which is used for swimming as well as for adsorption. Secondly inducible lateral flagella which are involved in swarming. Since the biosynthesis of flagella is NtrA (or RpoN)-dependent, chemotaxis is also dependent on this sigma factor.

In their hands, the best chemo-attractants of *Azospirillum brasilense* are arabinose, fucose and galactose. Chemotaxis towards these compounds is inducible. The gene *chvE* may be involved in chemotaxis since *chvE-lacZ* fusions are also induced be these sugars (J. Vanderleyden, personal communication).

Besides chemotaxis, azospirilla are capable of positive chemokinesis, i.e. the cells increase their speed when moving towards attractants (Zhulin and Armitage, 1993). Like in *Rhizobium*, no dedicated receptors have been found in *Azospirillum*. It has been suggested that these bacteria have a simple chemosensing system of which the main chemosensory pathway is based on metabolism of chemo-effectors (Zhulin and Armitage, 1993). Sensing of the proton motive force is probably involved in signal transduction during *Azospirillum* taxis (Zhulin et al., 1995). *Azospirillum* shows aerotaxis, i.e. it responds chemotactically towards low oxygen concentrations (Barak et al., 1982) and it is also capable of redox taxis, e.g. movement towards optimal redox potential (Grishanin et al., 1991). This response may be relevant for the rhizosphere where bacteria experience different concentrations of oxygen, ions and other chemicals that affect redox potential.
In soil, *A.brasilense* cells are capable of moving up to 30 cm from the inoculation point towards plant roots (Bashan and Levanony, 1987). Root - to - root travel is an active

process since a non-motile mutant did not show this behaviour (Bashan and Holguin, 1994).

Intercellular communication and small bacteriocin

Although toxins in general are not designated as signal molecules, it was shown that the rhizobial toxin small bacteriocin belongs to a class of signal molecules, the acyl-homoserine lactones (A-HSLs). Growth of *R.leguminosarum* bv. *viciae* strain 248 can be inhibited by 'small' (Hirsch, 1979). This strain and other strains car-rying a self-transmissible Sym-plasmid do not produce 'small'. Their Sym-plasmid contains the *sbs* gene, encoding 'small' bacteriocin sensitivity, as well as the *rps* gene, which represses the production of 'small'. 'Small' is typical for *R.leguminosarum* and is produced by strains of all three biovars of this species: *viciae, trifolii,* and *phaseoli*. 'Small' was purified and shown to be an A-HSL, a family of secreted molecules which activate gene expression related to intercellular communication at high cellular density (reviewed by Fuqua et al., 1994). 'Small' was identified as N-(3R-hydroxy-7-cis-tetradecanoyl)-L-HSL (Schri psema et al., submitted). Other A-HSLs are auto-inducers of *Vibrio fischeri* and *V.harveyi* - which activate *lux* gene expression (leading to luciferase production) - and conju-gation factor of *Agrobacterium tumefaciens* which is required for expression of the *tra* genes. Using an *A.tumefaciens tra-lacZ* reporter strain, 'small' appeared to induce ß-galactosidase significantly. Surprisingly, the supernatant fluid of the 'small'-negative *R.leguminosarum* strain 248 strongly induced ß-galactosidase. The latter inducer was purified and appeared to be another A-HSL, tentatively identified as hexanoyl-HSL (M. Schulte et al., unpublished). In conclusion, *R.leguminosarum* can produce at least two different A-HSLs whose functions are not yet known. Perhaps the toxic activity of 'small' has evolved from a molecule involved in density-regulation. Alternatively, one or both rhizobial A-HSLs may be autoregulators involved in processes such as conjugation (Wijffelman et al., 1983; Schripsema et al., 1994) and/or the transcription of the *rhi* operon (Dibb et al., 1984). Expression of Rhi protein requires the *rhiABC* operon as well as the linked *rhiR* gene. RhiR shows homology to LuxR but a LuxI homolog was not identified (Cubo et al., 1992). The Rhi protein is characteristic of *R.leguminosarum* bv. *viciae*, since it was not found in *R.leguminosarum bv. phaseoli* and in only one of the six strains of *R.leguminosarum bv. trifolii* tested. It is a 24 kDa protein which accumulates in the stationary phase in free-living cells but was not detected in bacteroids. Consistent with being density-regulated, Dibb et al. (1984) noted that the quantities of Rhi protein were much greater in cultures grown on solid medium than in liquid medium.

Synthesis of nod gene products

Experiments reported by Van Brussel et al. (1986) indicated that the basis of nodulation is a signal exchange between bacterium and plant: root exudate induces *nod* gene activation which results in secretion by the bacterium of factors which induce nodulation-related

processes in the plant. We now know that the *nod* gene inducer is often a flavonoid and that the secreted components are LCOs.
The general pattern of this process is that a bacterium contains one or more constitutive regulatory *nodD* genes and several inducible *nod* operons. *nodD* is usually activated by a flavonoid but betaines such as trigonelline and stachydrine, non-phenolic compounds such as hydroxycinnamic acids, and monocyclic aromatic compounds such as vanilline and isovanillin, have also been described as *nod* gene inducers (reviewed by Göttfert, 1993).

Individual *nodDs* differ in their flavonoid specificity, suggesting a direct contact between *nodD* and inducer. Direct biochemical proof for this notion is lacking. Nodulation of mung bean, cowpea and siratro by *B.japonicum* may involve another signal because the *nodVW* genes are required. *nodV* is most likely a sensor kinase responding to an as yet-unidentified external stimulus, while *nodW* is a response regulator (Göttfert, 1993). Lerouge et al. (1990) were the first who showed that the secreted product of Nod proteins is an LCO. It is now clear that the general structure of these final products of Nod proteins consists of a chitin oligomeric backbone with an N-linked fatty acyl residue on the non-reducing terminal residue. Specific modifications of, or substitutions to, this structure determine the host range of LCOs (reviewed by Carlson et al., 1994, and Bloemberg et al., *in press*). Although in general LCOs are secreted, LCOs in *R.leguminosarum bv. trifolii* have been reported to reside predominantly within the cell (McKay and Djordjevic, 1993) or to be bound to the membrane fraction (Orgambide et al., 1994a). Production and/or secretion of these LCOs has been reported to depend on environmental conditions including temperature, pH, phosphate and nitrate concentrations (McKay and Djordjevic, 1993). Spaink et al. (1995) observed that the difference between the LCOs of *R.leguminosarum* bvs. *trifolii* and *viciae* is in the hydrophobicity of the highly unsaturated fatty acyl chains and have suggested that the more hydrophobic nature of the *nodE*-dependent bv. *trifolii* fatty acyl chains is the major host range-determining factor between these biovars (Bloemberg et al., 1995). It is not clear yet why a certain *Rhizobium* strain often produces a series of different LCOs whereas signals are usually very specific. Moreover, it remains to be established how the LCO's interact.

When LCOs are added to plants, effects have been observed on root hairs and outer and inner cortical cells. These biological effects of LCOs have been described in recent reviews by Schultze et al. (1994) and Bloemberg et al. (*in press*). Briefly, LCOs induce root hair deformation, pre-infection threads, and cortical cell divisions leading to the formation of nodule primordia and sometimes to meristem formation. At the physical and molecular levels, LCOs depolarize the root hair membrane, increase proton flux, cause oscillating patterns of Ca^{2+} influx and efflux and cause rearrangements in the actin filament network. Furthermore, they activate early nodulin genes (ENOD5, ENOD12 and ENOD40) and cell cycle genes (*cdc*2Ms, *cyc*Ms2, histone H3-1), and cause activation of phenylalanine ammonia lyase and chalcone synthase. Recently, peroxidase induction has been added to this list (Cook et al., 1995). With respect to the nodulins, two recent observations must be mentioned. Firstly, work of Csanádi et al. (1994) showed that ENOD12 is neither required for nodulation, nor for nitrogen fixation. Secondly, *Medicago trunculata* ENOD40 DNA shows no significant open reading frame. Evaluation of the RNA secondary structure revealed a high degree of stability. This and further evidence suggested that the untranslated RNA acts as a ribo-regulator associated with growth control and differention (Crespi et al., 1994; Asad et al., *in press*).

LCOs are not the only reported nodulation-related signals that are produced upon activation of the *nod* genes. Firstly, Philip-Hollingsworth et al. (1991) showed that N-acetylglutamic acid (also designated as BF-5), which is secreted by *R.leguminosarum* bv. *trifolii* upon activation of *nodD*, induces a number of morphological responses on axenic roots of white clover seedlings. Significantly less of the signal is secreted by mutants lacking *nodL* or *nodM*. Since NodL is an O-acetyl-transacetylase and since NodM is related to genes encoding certain amidotransferases that use glutamine as amino donor and glutamate as by-product, it was speculated that these enzymes may play a direct role in the biosynthesis of a fraction of the secreted compound. Secondly, Orgambide et al. (1994b) showed that a diglycosyl diacylglycerol, also designated as BF-7, is produced by *R.leguminosarum* bv. *trifolii* in a *nod* gene inducer-, *nodD*-, and *nodABCIJT*-dependent way. Subnanomolar concentrations of the compound elicit a number of symbiosis-related morphological responses on white clover roots.

How do LCOs act at the molecular level? The search for receptors has just begun (Bono et al., 1995). Receptors will prove very difficult to identify. (i). LCOs are amphipathic molecules which show a high degree of aspecific binding. (ii). La-beled LCOs have to be used because LCOs are active in low concentrations. Care should be taken that the labeled molecules of such a preparation are still biologically active. (iii). LCOs can be rapidly degraded by the plant. When the LCO has been labeled at a specific position one runs the risk of following the fate of a by-product. In this respect it is interesting to note that O-acetylated chitin fragments, applied by microballistic targeting into root cortical cells together with the stele factor uridine (Smit et al., submitted), are able to induce nodule primordia. This suggests that the acyl moiety of LCO is involved in transport of the active part of the molecule to the inside of the plant (Spaink et al., 1994).

INFECTION AND HOST DEFENCE

The high density of bacteria in the infection thread suggests to us that the situation in this ecological niche is ideal for expression of density-regulated genes as well as for, so far hypothetical, oxygen limitation-regulated processes.

R.meliloti EPS-I (succinoglycan) mutants produce pseudo-nodules on alfalfa. Their infection thread does not penetrate beyond the epidermal cell layer. In contrast to the wild type, EPS-I mutants induce a plant defence response (thickened cell walls containing callose and phenolic compounds; increased PAL and peroxidase) (Niehaus et al., 1993). This could just be the result of poor infection. However, the addition of a tetramer of the succinoglycan repeat unit, fully substituted with succinyl- and pyruvyl groups, restores nodule invasion by *R.meliloti exo* mutants (Battisti et al., 1992). The latter result suggests an active role for EPS-I in nodule development, presumably by suppressing the plant defence response. It should be noted that wild type *R.meliloti* can also elicit a defence mechanism, similar to the hypersensitive reaction (Vasse et al., 1993).

Low concentrations of purified *R.leguminosarum* bv. *trifolii* LPS were reported to promote infection thread formation in white clover root hairs, whereas this process was inhibited by a higher dose (Dazzo et al., 1991). Results with O-antigen defec-tive mutants are consistent with an effect of LPS on the formation of proper infection threads (Noel et al., 1986; Stacey et al., 1991) in *B.japonicum* and *R.leguminosarum* bv. *phaseoli*, both of which form determinate (round) nodules. In the case of *R.leguminosarum* bv. *trifolii* and bv. *viciae*, which form indeterminate (continually elongating) nodules on clover and pea, respectively, the mutant bacteria proceed through infection threads. Bacterial release from the infection thread is impaired but the cells are eventually released into the host cell, resulting in Nod$^+$ Fix$^-$ nodules in which no or very few plant cells contain bacteroids (De Maagd et al., 1989; Priefer, 1989). Tissue and cell invasion of the mutant bacteria is often associated with a host plant defence reaction. These results may suggest a signalling role for LPS or an LPS-derivative in the avoidance of host cell defence reactions during the invasive phase of nodule development (Perotto et al., 1994). Alternatively, they may simply be the result from poor infection.

Purine auxotrophs of *R.etli* CNF42 (bv. *phaseoli*) elicit pseudonodules on bean plants. The mutants cause Had (hair deformation) and cortical cell division but do not elicit infection threads and no bacteria can be isolated from the resulting pseu-donodule. Addition of AICA riboside, the unphosphorylated derivative of the purine precursor 5'P-ribosyl-5-amino-4-imidazole carboxamide (AICAR), leads to the formation of infection threads, but the number of bacteria recovered from the nodules is still at least 100-fold lower than for the wild type. Since AICA riboside is a very poor purine source for the strains, the results suggest that AICAR plays a role in infection thread development, presumably by the conversion to a factor in a pathway that deviates from purine biosynthesis (Newman et al., 1994).

HORMONES AND NODULATION

This topic has recently been reviewed by Hirsch and Fang (1994). Since the role of hormones in nodulation is not clear, the topic will be treated briefly. Auxin, cytokinin, gibberellin and ABA can be isolated from nodules. It is not clear which of the symbiotic partners is the source of the hormones. Ethylene is a potent inhibitor of nodulation. Non-nodulating pea mutants, which are blocked at the nodule primordium stage, can be restored to normal development if treated with AVG (amino-ethoxyvinylglycine) or Ag^+, which inhibit ethylene formation and action, respectively (Guinel and LaRue, 1991). Similarly, the nodulation ability of some exo-mutants of *R.leguminosarum* bv. *viciae* could be restored by minimizing ethylene production with AVG (van Workum et al., 1995). Hirsch et al. (1989) have shown that nodule primordia and early nodulins are induced by auxin transport inhibitors. Cooper and Long (1994) have recently shown that the Nod-minus phenotype of *Rhizobium* nodulation mutants can be suppressed by the *Agrobacterium tumefaciens* trans-zeatin secretion (*tzs*) gene. All these results indicate that a shift in the hormonal balance is crucial for nodulation.

Autoregulation of nodulation, i.e. the control by the plant of the number and size of its nodules, is lacking in supernodulating legume mutants. The autoregulatory factor of soybean appeared to be shoot-derived (Caetano-Anolles and Gresshoff, 1991). Autoregulation can also be triggered by LCOs (Van Brussel et al., 1992). Libbenga et al. (1973) showed that cell divisions occur in pea root cortical explants in the presence of cytokinin and auxin at positions where nodules would emerge. This response could be enhanced by stele factor(s), (one of) which was recently identified as uridine. The stele factor can be inactivated by Nod^+ rhizobia and mitogenic Nod factors in a normal pea plant, but not in a supernodulating Nod3 pea mutant whose parent produces a root-derived autoregulatory factor (Smit et al., 1993). This suggests that inactivation of the stele factor may be part of the autoregulatory response, and could influence production of the root-derived factor.

Do LCOs mimick endogenous plant hormones? It has been hypothesized that LCOs mimick endogenous plant signal molecules involved in developmental processes. (i). Transgenic *nodAB*-containing *Nicotiana* plants are severely altered in growth and development (Schmidt et al., 1993). (ii). LCOs are able to rescue a *Daucus* somatic embryo mutant that can also be rescued by the external application of a 32 kDa endochitinase derived from wild type embryos (De Jong et al., 1992; De Jong et al., 1993). (iii). *Medicago* can form nodules spontaneously, in the absence of rhizobia (Truchet et al., 1989). (iv). A correlation exists between the expression of chitinases and developmental processes (Collinge et al., 1993). (v). Recent results indicate that lipophilic chitin derivatives are present in plants (see Bloemberg et al., 1995). Since chitinous tissue is present in the fish *Paralipophrys* (Wagner et al., 1993) and since NodC has a significant homology with *Xenopus* DG42 protein which is expressed during embryogenesis (Bulawa et al., 1991), LCO analogues may be widespread in nature. Indeed, it has recently been shown that the *Xenopus* DG42 protein is able to direct the synthesis of compounds which share many characteristics with chitin oligosaccharides (Semino and Robbins, 1995).

NITROGEN CONTROL

Inhibition of nodule formation and nitrogen fixation has been reviewed by Streeter (1988), Carroll and Mathews (1990), and Schultze et al. (1994). Combined nitrogen exerts its effects at multiple stages. It has been reported to inhibit root hair deformation, bacterial adhesion and infection thread formation. At the molecular level, EPS synthesis is decreased by the presence of ammonia. Furthermore, auxin and ethylene may be involved in the control of nodulation by nitrogen. IAA partially releaves the effects of nitrate on the early events of infection. Moreover, AVG reduces the nitrate inhibition of nodulation, suggesting that ethylene may mediate this control. Recently, Coronado et al. (in press) have shown that nitrogen limitation of *Medicago sativa* results in enhancement of the expression of flavonoid biosynthetic genes, and in increased amounts of flavonoids and isoflavonoids in the root and of *nod*-gene inducing activity in the root extract. During evolution rhizobia may have started to use this increase in flavonoid production as a symbiotic signal for interacting with the nitrogen-starved plant (Coronado et al., in press). Van Brussel et al. (1990) showed that incubation of *Vicia* with its symbiotic partner resulted in a 10-fold increase of *nod* gene inducers in the root exudate (INI, increased nodulation gene inducing activity). Dokora et al. (1993a and 1993b), showed that alfalfa and bean respond to their symbiotic bacteria by exuding medi-carpin, daidzein and coumestrol, whose production is normally elicited by pathogens. Their results can be interpreted as indicating that the first response of the plant to *Rhizobium* is like that towards a pathogen, and that rhizobia use the re-sulting products as a trigger for inducing symbiotic *nod* genes. Some of the genes acting at different stages of symbiotic development are reg-ulated by combined nitrogen. Inhibition by nitrate is primarily controlled by the host plant (Carroll and Mathews, 1990). Ammonia, the primary product of nitrogen fixation, regulates bacterial *nod* gene expression, namely: *nodD3* and *nodABC* in *R.meliloti* (Dusha et al., 1989), and *nodD* and *nodYABC* in *B.japonicum* (Wang and Stacey, 1990). In contrast, the *nodD* and *nodABC* genes of *R.leguminosarum* bv. *viciae* are not regulated by combined nitrogen (Baev et al., 1992).

In *R.meliloti* the situation is as follows (Dusha and Kondorosi, 1993). NodD1 activates the *nodABC* promoter and is required for nodulation. NodD3 requires *syrM* for expression and participates in *nodABC* activation. Ammonia affects bacterial *nod* gene expression through the Ntr general nitrogen regulation system. At high ammonia concentrations NtrR negatively regulates *syrM*, thereby de-creasing expression of *nodD3*, thus also of *nodABC*. At low ammonia concentrations NtrC positively regulates expression of *nodD3* and therefore of *nodABC*.

REGULATION OF NITROGEN FIXATION

This topic has recently been reviewed extensively by Fischer (1994) and Batut and Boistard (1994). In *Klebsiella pneumoniae* environmental nitrogen and oxygen conditions are critical signals for the regulation of *nif* gene expression. Dual metabolic control is mediated via the Ntr general nitrogen regulatory system and the *nif*-specific regulatory genes, *nifLA*. By contrast, expression of nitrogen fixation genes in symbiotic diazotrophs is regulated predominantly in response to cellular oxygen concentrations. Oxygen concentrations in the infected zone of a pea nodule are below 30 nM whereas 250 µM of dissolved oxygen is measured in the air outside the nodule. The present status of the control of *nif* and *fix* gene expression in *R.meliloti* will briefly be treated here. The first oxygen control is on nitrogenase activity: nitrogenase is irreversibly inactivated by oxygen. The second control is on nitrogenase synthesis at the transcriptional level so that nitrogenase synthesis is prevented under oxygen conditions which are not compatible with nitrogenase activity. Low oxygen results in autophosphorylation of the FixL sensor which activates the FixJ transcriptional regulator by phosphorylation, which in turn increases *fixK* and *nifA* transcription. FixK is required for activation of the *fixN* promoter, leading to production of microaerobically-induced, membrane-bound cytochrome oxidase. The NifA tran-scriptional regulator is required for transcription of the *nifHDK* structural nitro-genase genes. *nifA* transcription is also increased by low O2. The *nifA* promoter is dependent on the sigma factor RpoN. Finally, low nitrogen also increases *nifHDK* transcription through NtrC.

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NODULATION IN LEGUMES AND ACTINORHIZAL PLANTS

GUAN C., PAWLOWSKI K. AND BISSELING T.
Department of Molecular Biology, Agricultural University, Dreijenlaan 3,
6703 HA Wageningen, The Netherlands

Introduction

All nitrogen in living organisms is ultimately derived from atmospheric dinitrogen which gets incorporated into organic compounds by biological or chemical nitrogen fixation. Since biospheric nitrogen is subjected to a rapid turnover by denitrification, maintenance of the biosphere has to be achieved by nitrogen fixation. Biological nitrogen fixation is an energy-consuming process performed by the enzyme nitrogenase which is irreversibly denatured by oxygen. Nitrogenase is formed only by prokaryotes who in some cases fix nitrogen in symbiosis with higher plants. In these symbioses, bacteria are hosted inside plant cells in special organs, the so-called root nodules. The product of nitrogen fixation, ammonium, is exported to the plant, while the plant in turn is providing its symbiont with energy sources. Of the symbiotic nitrogen fixers, two distinct phylogenetic groups are (*Azo-*, *Brady-*)*Rhizobium* and *Frankia* who fix atmospheric nitrogen in association with higher plants, leading to the *Rhizobium*-legume symbioses and *Frankia*-actinorhizal symbioses (Young, 1992). *Rhizobium* enters symbioses only with leguminous plants (with the exception of *Parasponia*; Trinick, 1979), while *Frankia* is able to nodulate a taxonomically diverse group of plants which recently have been found to be closely related amongst each other and with legumes (Soltis et al., 1995). These plants are collectively referred to as actinorhizal plants.

Leguminous root nodules represent stem-like structures with peripheral vascular bundles and infected cells in the central tissue. Two types of leguminous root nodules have been defined, the determinate and the indeterminate nodules. Indeterminate nodules are characterized by a persistent distal meristem (Newcomb, 1976). Due to its activity, a developmental gradient from the meristem to the proximal senescence zone is present in the central tissue of the nodule, by which the central tissue can be divided into specific zones (Vasse et al., 1990; Fig. 1A). The meristem (zone I) is followed by the prefixation zone (zone II), where infection of the cortical cells takes place. In the so-called interzone II-III, bacterial nitrogen fixation is induced (Yang et al., 1991) and proceeds throughout the nitrogen fixation zone (zone III). In the senescence zone (zone IV), bacteroids are degraded by the plant. Determinate nodules do not have a persistent meristem (Newcomb et al., 1979). The nodule meristem ceases to divide at an early stage of development. As a result, all the cells of the central tissue are at a similar stage of development at any given time point.

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The presence of nodular structures (actinorhizae) on the roots of actinorhizal plants was first reported for alder in 1829 (Meyen, 1829). Since then, actinorhizae have been found among over 25 genera of dicotyledonous plants belonging to eight different families (Benson, Silvester, 1993). Actinorhizal root nodules display an indeterminate growth pattern (Fig. 1B). In contrast to legume nodules, they represent coralloid structures composed of several modified lateral roots without root caps (lobes). Actinorhizal nodule lobes contain a central vascular bundle, and infected cells in the cortex (reviewed by Silvester et al., 1990). In some cases, the nodule lobe meristems differentiate agraviotropically growing roots in stead of root caps. These "nodule roots" contain large air spaces and serve to aerate the nodule tissue.

The interaction between host plants and microsymbionts starts with signal exchange and recognition of the symbiotic partners. From this step until nodule formation and functioning, many genes from both partners participate in the process. Some of them are nodule-specific plant genes, called nodulin genes (Van Kammen, 1984) in *Rhizobium*-legume symbioses, and actinorhizin genes in actinorhizal symbioses (Tremblay et al., 1986). Nodulins are grouped into early and late nodulins based on the time course of their expressions (Nap, Bisseling, 1990).

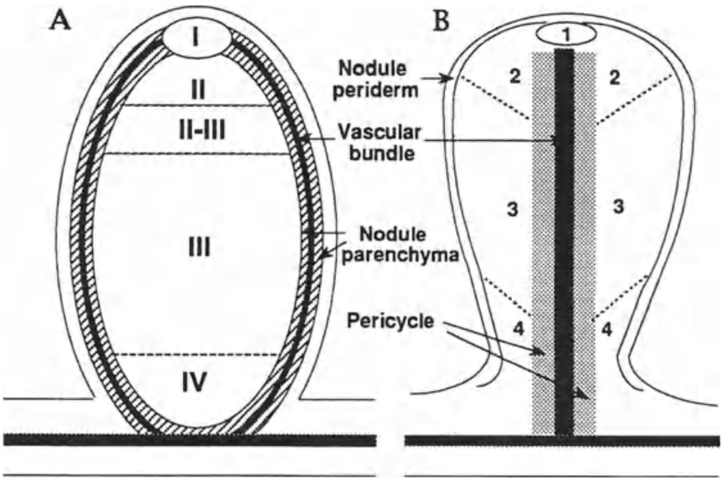

Figure 1. Comparison of an indeterminate legume nodule and an actinorhizal nodule.

(A) Legume nodules contain a peripheral vascular system and infected cells in the central tissue. An O_2 diffusion barrier is present in the nodule parenchyma surrounding the nodule vascular bundles. Since the nodule parenchyma is interrupted by the meristem (zone I), an O_2 gradient forms that extends from the distal to the proximal end of the nodule. Due to the activity of the meristem, the cells in the central tissue are arranged in a developmental gradient (see text). II, prefixation zone; II-III, interzone; III, fixation zone; IV, senescence zone.

(B) Actinorhizal nodule lobes contain a central vascular bundle and are surrounded by a periderm. Due to the activity of the distal nodule meristem (1), a developmental gradient is formed in the cortex. In the fixation zone (2) cortical cells enlarge and some of them are infected and gradually filled by *Frankia* hyphae. In the fixation zone (3) *Frankia* vesicles have formed and nitrogen fixation (*nif*) genes are expressed, resulting in nitrogen fixation. In the senescence zone (4), plant and bacterial material is degraded in the infected cells.

Nodule induction and Nod factors

Nodule development

In most cases, infection of legume plants by rhizobia starts by root hair deformation (Bauer, 1981). While rhizobia attach to the root hair, the root hair deforms as a response to rhizobial Nod factors (Lerouge et al., 1990; Heidstra et al., 1994), and the bacteria get trapped in the root hair curl. By hydrolyzing the local root hair cell wall (Callaham, Torry, 1981), the bacteria enter the epidermal cell in an infection thread which proceeds to grow through the cortical cells. Simultaneously, cortical cells are mitotically activated, giving rise to the nodule primordium (Dart, 1977). Infection threads grow toward the primordium and then the bacteria are released into the cytoplasm of the host cells, surrounded by a plant-derived peribacteroid membrane (PBM). The nodule primordium thereupon develops into a mature nodule, while the bacteria differentiate into their endosymbiotic form, bacteroids (Fig. 2A). At this stage, bacteroids synthesize nitrogenase which catalyzes the reduction of nitrogen. In a few cases, however, infection follows a so-called crack-entry mechanism without formation of infection threads (Chandler, 1978).

Frankia can infect its host plants in two different ways, root hair infection or epidermal intercellular penetration. Which of these ways is realized depends on the plant species (Miller, Baker, 1985; Racette, Torry, 1989). In root hair infections, *Frankia* hyphae are entrapped in the deformed root hair. The trapped *Frankia* hyphae branch and initiate the digestion of the primary cell wall of the root hair, and the host plant in turn begins to build an additional cell wall around the site of digestion (Callaham et al., 1979; Berry et al., 1983). Continued digestion by the bacteria and triggered wall building by the host plant create a tubular ingrowth which is termed encapsulation (Lalonde, Knowles, 1975; Newcomb, Wood, 1987). This structure is analogous to the infection thread in *Rhizobium*-legume symboses. Mitotic activity in the root cortex and cell expansion result in the formation of the prenodule whose cells are infected by encapsulated *Frankia* hyphae. Subsequently, one or several nodule lobe primordia are initiated in the root pericycle. *Frankia* hyphae grow from the root cortex into the developing nodule lobe primordia through cortical cells. Upon entering the primordia, once again, *Frankia* hyphae branch and invade numerous cells of the young nodule cortex, being surrounded by encapsulating material derived from the host cell wall as well as by the host plasma membrane (Fig. 2B). Infected cells are filled with hyphae from the center outward; then *Frankia* begins to produce differentiated vesicles where nitrogenase is induced and nitrogen fixaion starts (reviewed by Berry, Sunell, 1990). Vesicles are terminal swellings or differentiate from short branch hyphae. In contrast to *Rhizobium*, *Frankia* can fix nitrogen also in the free-living state under atmospheric oxygen tension by forming these specialized vesicles (Benson, Silvester, 1993). In the vesicles, nitrogenase is protected from oxygen by the multilayered lipid envelope (Berry et al., 1993).

Rhizobium Nod factors and Frankia factor

The so-called Nod factors are *Rhizobium*-derived lipo-chitooligosaccharides, that play a key role in the induction of the initial stages of nodulation. The bacterial genes involved in Nod factor synthesis are the *nod* (nodulation) genes. These genes are not expressed in free-living bacteria, with the exception of *nodD*, which is expressed constitutively. Its product NodD is able to bind to specific flavonoids secreted by the roots of the host plant (Goethals et al., 1992); upon binding to flavonoids, it acts as a transcriptional activator of the other *nod* genes (Fisher, Long, 1992), which encode enzymes involved in the synthesis of Nod factors. The structure of a Nod factor from *R. meliloti* was first determined in 1990 (Lerouge et al., 1990) and since then Nod factor structures have been determined from other rhizobia (Van Rhijn, Vanderleyden, 1995). In general, Nod factors consist of a backbone of three to five β-1,4-linked *N*-acetylglucosamines bearing a fatty acid on the non-reducing sugar residue. Furthermore, the factors can have various substitutions on the reducing and the non-reducing terminal sugar residues.

Due to the striking similarities of the initial infection steps between *Rhizobium*-legume symbioses and the *Frankia*-actinorhizal symbioses, similar signaling mechanisms between the microsymbionts and the hosts are expected. However, investigations to detect any sequences homologous to the *nod* genes in the *Frankia* genome have failed so far (Simonet et al., 1990; Chen et al., 1992). This might mean that the *Frankia nod* genes are not conserved at the DNA level, but proteins with the corresponding functions may exist in *Frankia*. However, no functional complementation has been observed yet (Chen et al., 1992; Reddy et al., 1992). In most recent studies, it was found that (a) *Frankia* factor(s) eliciting root hair deformation on host plants is released into the growth medium, and is produced constitutively by *Frankia* strain ArI3 (Van Ghelue, 1994). Culture filtrate of *Frankia* strain ArI3 only led to deformation of the root hairs of *Alnus glutinosa* and not of non-host plant species tested, indicating a degree of specificity. So far no root hair deformation factors from *Frankia* have been purified and their chemical properties remain unknown.

Gene induction by Nod factors

Rhizobium Nod factors can induce the expression of several plant genes in the epidermis of legume roots (Vijn et al., 1995). The early nodulins *ENOD5* (Scheres et al., 1990b) and *ENOD12* (Scheres et al., 1990a), which encode proline-rich proteins, and *Mtrip1* (Cook et al., 1995), which encodes a peroxidase, represent such genes. The latter gene is expressed in the root pericycle of uninoculated roots; all three genes are induced in the epidermis within a few hours after application of Nod factors (Horvath et al., 1993; Journet et al., 1994; Cook et al., 1995). The induction of *ENOD12* and *Mtrip1* expression occurs in a relatively broad zone of the root, starting just above the root tip, where root hairs have not yet emerged, and extending to the region containing mature root hairs (Pichon et al., 1992; Cook et al., 1995). Cytological studies have shown that Nod factors elicit the expression of these genes in all epidermal cells (Journet et al., 1994; Cook et al., 1995), and that a direct contact between Nod factors and epidermal cells is required (Journet et al., 1994). Moreover, Nod factors can induce nodulin gene expressions in the cortical cells which are mitotically reactivated to form the nodule primordium. *ENOD12* and *ENOD40* represent such genes (Vijn et al., 1993). Furthermore, *ENOD40* is also induced by Nod factors in the root pericycle (Kouchi, Hata, 1993; Yang et al., 1993; Asad et al., 1994). This gene has a phytohormone effect when expressed in the non-legume tobacco (K. Pawlowski, R. Walden, personal communication).

Onset of nitrogen fixation

Symbiosis-specific differentiation

In *Rhizobium*-legume interactions, the intracellular bacteria differentiate into their symbiosis-specific form, the bacteroids, after release from the infection thread into nodule primordium cells (Fig. 2A). Because both plant (Häser et al., 1992) and bacterial (Glazebrook et al., 1993) mutants have been identified that are specifically defective in bacteroid differentiation, this process may be independent of internalization of bacteria by the infected cells. Bacterial mutants specifically defective in the release of bacteria from the infection thread are known as well (De Maagd et al., 1989). Bacterial *nod* genes are expressed in the distal part of the prefixation zone II (Schlaman et al., 1991), indicating that Nod factors may play a role in signal exchange within the nodule. However, since bacterial release and bacteroid development can be impaired in bacterial strains with functional *nod* genes, other bacterial and/or plant signals must also play a role in these steps of development.

In all the actinorhizal genera except *Casuarina* and *Allocasuarina*, the onset of nitrogen fixaton is associated with the appearance of *Frankia* vesicles in the infected host cortical cells (Berry, Sunell, 1990). Vesicles are also formed in free living *Frankia* cultures under aerobic conditions when substrate nitrogen is limiting (Tjepkema et al., 1980). Within the cytoplasm of the vesicle, septations may occur which divide the cell into compartments (Fig. 2B). The function of these septa is unknown. Within nodule tissue the extent of vesicle formation, the shape and the spatial organization of the vesicles are controlled by the host plant. Symbiotic vesicles may be spherical, club-shaped, elliptical, or filamentous (Newcomb, Wood, 1987; Racette, Torrey, 1989). Thus, like *Rhizobium*, *Frankia* shows some symbiosis-specific differentiation.

Establishment of the interface between the partners

Root nodules provide a proper environment to allow efficient nitrogen fixation by the microsymbiont and regulated nutrient exchange between both symbionts. The nutrient exchange is regulated by occurrence of plant-derived membranes that in all cases surround the "intracellular" microsymbiont. In legume nodules, bacteroids are enclosed in peribacteroid membranes (PBMs; Fig. 2A). They form the interface between the symbiotic partners across which signals and metabolites are exchanged and prevent a defense response by the plant against the "intracellular" bacteria (Nap, Bisseling, 1990; Verma, 1992; Werner, 1992). This process of endosymbiont internalization and propagation requires massive membrane synthesis, and in the case of soybean nodules equals 30 times the amount of plasma membrane synthesis (Verma, 1992).

The PBM of legume nodules has a phospholipid (Perotto et al., 1995) and protein composition that is different from that of the plasma membrane (Verma, 1992) and that (presumably) endows it with specialized functions. The PBM contains several plant proteins and may even contain a rhizobial protein (Fortin et al., 1985; Miao et al., 1992). Within the peribacteroid space (PBS) between the bacteroids and the PBM, several proteins are present that are also found in vacuoles, e.g. α-mannosidase II (Kinnback et al., 1987; Mellor, Werner, 1987), proteases (Mellor et al., 1984), and protease inhibitor (Garbers et al., 1988; Manen et al., 1991). Thus, the PBM may have adapted some properties of the tonoplast membrane (Mellor, Werner, 1987). Indeed, it has been proposed that the symbiosome (the PBM with enclosed bacteroids) has properties of a lytic compartment that is continuously being neutralized by ammonia exported by the bacteroids (Kannenberg, Brewin, 1989). According to this hypothesis, one would expect that the lack of bacterial nitrogen fixation would lead to bacteroid degradation. In fact, there is evidence for premature bacteroid degradation of non-fixing *Rhizobium* mutants (e.g. Hirsch, Smith, 1987). Because the PBM constitutes the interface between bacteroids and host plants, it plays an important role in controlling the exchange of metabolites. These metabolites include ammonium, the product of nitrogen fixation, and heme, the prosthetic group of the oxygen transport protein leghemoglobin, which are exported by the bacteroids to the host cytoplasm (Nadler, Avissar, 1977; O'Gara, Shanmugan, 1976), and carbon sources and probably also assimilated ammonium, which are supplied by the host to the bacteroids (De Bruijn et al., 1989; Werner, 1992). Which proteins are involved in the transport of these compounds is largely unclear. Nodulins Ngm-26 and Ngm-23 have been localized in the peribacteroid membrane and have been supposed to play a role in PBM structure or function (Jacobs et al., 1987; Sandal et al., 1987; Ouyang et al., 1991).

In actinorhizal nodules, the interface between the microsymbiont and the host plant is the invaginated plasma membrane formed around the growing hyphae. Within this membrane, *Frankia* is still surrounded by the pectinaceous cell wall-like encapsulation. It is not clear yet if nodule-specific plant proteins are incorporated into the invaginated plasma membrane. However, it has been postulated that the product of a nodule-specific cDNA in *Alnus glutinosa*, *ag12*, which encodes a subtilisin-like protease with a putative signal peptide, might be involved in the processing of a protein that is part of the encapsulation material surrounding the bacteria or of another protein with an undeterminated function (Ribeiro et al., 1995; Fig. 3-C, 3-D). The *ag12* transcript was found mainly in the newly infected cells of zone 2 in the nodule where nitrogen fixation has not started yet.

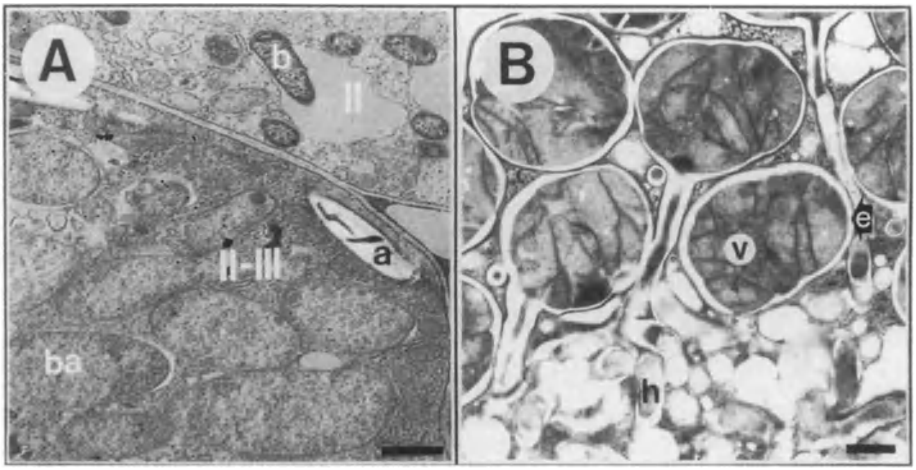

Figure 2. Intracellular nitrogen-fixing bacteria. (A) Intracellular rhizobia in a nodule formed on clover by *R. trifolii*. A magnification of the transition of the prefixation zone II to the interzone II-III is shown. In the cell of zone II, intracellular bacteria (b) have not yet differentiated into their nitrogen-fixing form. In the cell of interzone II-III, which contains amyloplasts (a), nitrogen-fixing bacteroids (ba) have differentiated. Intracellular bacteria are surrounded by the plant-derived peribacteroid membrane (PBM). The photograph was kindly provided by U. Bialek and A. van Lammeren.

(B) Intracellular *Frankia* in a nodule of *Alnus serrulata*. Vegetative hyphae (h) and nitrogen-fixing septate vesicles (v) can be seen. Vesicles are surrounded by a lipid envelope (arrow) that provides O_2 protection of the nitrogen fixation process. Both hyphae and vesicles are surrounded by encapsulating cell wall-like material and the invaginated plasma membrane of the host cell. The photograph was kindly provided by R.H. Berg.

Nodule metabolism

Assimilation and transport of ammonium. - The form in which nitrogen is transported depends on the plant: temperate legumes, which generally form indeterminate nodules, export amides, whereas tropical legumes, which form determinate nodules, export ureides. Actinorhizal plants mostly export amides, with the exception of *Alnus* sp. and *Casuarina equisetifolia*, which are citrulline exporters (Schubert, 1986; Sellstedt, Atkins, 1991). In all cases, ammonium is exported by the microsymbiont as the first product of nitrogen fixation and is assimilated in the cytoplasm of nodule cells via the glutamine synthetase (GS)/glutamate synthase (GOGAT) pathway (Schubert, 1986). Subsequently, glutamate will be metabolized into nitrogen transport forms.

In legume nodules, the products of several late nodulin genes play a role in this metabolism. In ureide producing determinate legume nodules, the assimilation of ammonium by GS and the biosynthesis of ureides are spatially separated to some extent: whereas GS is expressed in both infected and uninfected cells of soybean nodules (Miao et al., 1991), uricase (nodulin-35), one of the key enzymes in purine oxidation which catalyzes the oxidation of uric acid to allantoin, has been found in peroxisomes of uninfected cells only (Hanks et al., 1981; Nguyen et al., 1985). Allantoinase, which is catalyzing the next step in purine oxidation, has also been localized in the uninfected cells (Hanks et al., 1981). The uninfected cells of determinate nodules also seem to be involved in transport of fixed nitrogen. The presence of GS in the infected cells of actinorhizal nodules of *Alnus glutinosa* has been confirmed by both enzymatic activity measurements and immunological studies (Hirel et al., 1982), and by *in situ* hybridization (C. Guan, A. Ribeiro, T. Bisseling and K. Pawlowski, unpublished results).

Carbon metabolism.- The carbon source transported from the leaves to the nodules is sucrose (Hawker, 1985), which is introduced into nodule metabolism through degradation by sucrose synthase. This enzyme is present at high levels in both legumes and actinorhizal nodules (Thummler, Verma, 1987; M. Van Ghelue, A. Ribeiro, A. Akkermans, B. Solheim, A. Van Kammen, T. Bisseling and K. Pawlowski, unpublished observations). In case of *Rhizobium*-induced nodules, bacteroids express a dicarboxylic acid uptake system. Isolated bacteroids take up dicarboxylic acids, and mutants in this uptake are symbiotically ineffective (Ronson et al., 1987; Werner, 1992), indicating that dicarboxylic acids are likely to be the carbon source supplied by the plant to the intracellular bacteria. It has been suggested that nodulin Ngm-26 transports the dicarboxylic acids to the bacteroids (Ouyang et al., 1991). However, its low substrate specificity *in vitro* indicates that it is more likely to form a pore responsible for the uptake of ions or small metabolites in general (Weaver et al., 1994).

The form of carbon that is supplied to symbiotic *Frankia* in actinorhizal nodules is not clear, yet a malate/aspartate shuttle between host and microsymbiont has been suggested (Akkermans et al., 1981). Based on the hypothesis, like in legume nodules, sucrose has to be metabolized to phosphoenolpyruvate (PEP) to provide the carbon source for the microsymbiont. Sucrose synthase or invertase and glycolytic enzymes would be involved. Subsequent carbon dioxide fixation by PEP carboxylase would lead to the formation of dicarboxylic acids which can be reduced to malate. High activity of PEP carboxylase have been found in legume (King et al., 1986) as well as actinorhizal nodules (McClure et al., 1983).

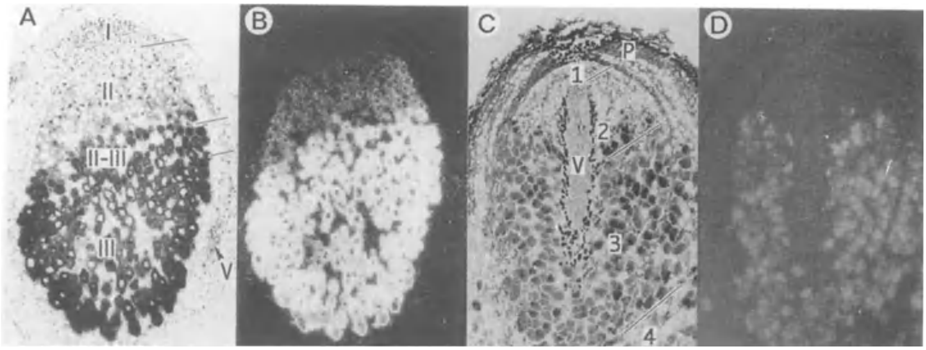

Figure 3. Nodule-specific gene expression.

(A,B) Leghemoglobin (*lb*) is expressed in zones II, II-III and III in a pea nodule. Thus, *lb* expression is preceding nitrogen fixation which starts in the interzone II-III. The photographs were kindly provided by W. C. Yang.
(C,D) Expression of *ag12* in a nodule of *A. glutinosa* is confined to the infected cells of zones 2 and 3. Highest expression levels are found in the infected cells of zone 2 where nitrogen fixation does not take place. *ag12* encodes a subtilisin-like protease.
(A,C) represent bright field micrographs where silver grains denoting hybridization are visible as black dots. The nodule vascular bundle (V) and periderm (P) are labeled (see also Fig. 1). In the dark field micrographs **(B,D)**, silver grains are visible as white dots.

Oxygen control

The enzyme nitrogenase is highly oxygen sensitive, because one of its components, the iron molybdenum cofactor, is irreversibly denatured by oxygen (Shaw, Brill, 1977). On the other hand, the large amount of energy required for this reaction has to be generated by oxidative processes; thus, there is a high demand for oxygen in nodules. Different strategies are used in different symbiotic interactions to cope with this paradox.

In legume nodules, a low oxygen tension in the central part of the nodule is achieved by a combination of a high metabolic activity of the microsymbiont and an oxygen diffusion barrier in the periphery of the nodule, that is, in the nodule parenchyma (Witty et al., 1986; Fig. 1A). Oxygen is supposed to diffuse via the intercellular spaces. The nodule parenchyma contains very few and small intercellular spaces where nodulin genes such as *ENOD2* are expressed whose protein products might contribute to the formation of the oxygen barrier (Van de Wiel et al., 1990). In the infected cells of the central part of the nodule, high levels of the oxygen carrier protein leghemoglobin facilitate oxygen diffusion to the sites of respiration (Fig. 3-A, 3-B). In this way, the microsymbiont is provided with sufficient oxygen to generate energy within a low overall oxygen concentration (Appleby, 1984). In contrast to *Rhizobium*, *Frankia* bacteria can form specialized vesicles in which nitrogenase is protected from oxygen (Berry et al., 1993; Benson, Silvester, 1993). However, vesicle formation during symbiosis does not take place in all *Frankia*-actinorhizal interactions (Benson, Silvester, 1993) and does not always seem to provide full oxygen protection of nitrogenase in others (Tjepkema, 1983; Kleemann et al., 1994). In *Casuarina* symbioses, an oxygen diffusion barrier is established around groups of infected cells by lignification of the walls of both infected and adjacent uninfected cells (Berg, McDowell, 1988; Zeng et al., 1989). In addition, the oxygen transport protein hemoglobin, the equivalent of leghemoglobin, is expressed in the infected cells of *Casuarina* nodules (Fleming et al., 1987; Tjepkema, Asa, 1987; Jacobsen-Lyon et al., 1995). Also in *Myrica* nodules, an oxygen diffusion barrier is present (Zeng, Tjepkema, 1994) and high amounts of hemoglobin have been found, although *Frankia* forms vesicles in this symbiosis (Tjepkema, Asa, 1987).

The leghemoglobin genes have been extensively studied in *Rhizobium*-induced symbioses. Promoter analysis of these genes has led to the identification of a so-called organ specific *cis*-acting element (OSE; Ramlov et al., 1993) which was also found in the promoter of the nodule-specific hemoglobin gene of the actinorhizal plant *Casuarina glauca* (Jacobsen-Lyon et al., 1995). A *C. glauca* hemoglobin promoter-GUS fusion was expressed in the infected cells of *Rhizobium*-induced nodules on *Lotus corniculatus* (Jacobsen-Lyon et al., 1995), implying that similar regulatory factors are involved in both legume and actinorhizal systems. This, in addition to the newly found phylogenetic relationship between different actinorhizal plant genera on the one hand and between actinorhizal plants and legumes on the other hand (Soltis et al., 1995), leads to the hypothesis that the ability for plants to enter symbioses is a trait which developed only once in evolution. Thus, actinorhizal and *Rhizobium*-legume symbioses seem to be closely related.

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EARLY EVENTS IN THE *AZORHIZOBIUM CAULINODANS—SESBANIA ROSTRATA* SYMBIOSIS

MERGAERT, P.[1], GOORMACHTIG, S.[1], GEELEN, D.[1], GEREMIA, R.[2], VALERIO-LEPINIEC, M.[1], FERNÁNDEZ-LÓPEZ, M.[1], GOETHALS, K.[1], D'HAEZE, W.[1], PROMÉ, J.-C.[3], DE BRUIJN, F.J.[4], VAN MONTAGU, M.[1], and HOLSTERS, M.[1]

[1]Laboratorium voor Genetica, Universiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium; [2] Centre de Recherches sur les Macromolécules Végétales, F-38041 Grenoble Cedex, France; [3] Laboratoire de Pharmacologie et Toxicologie Fondamentales, F-31077 Toulouse, France; [4] MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824, USA

The symbiotic interaction between the tropical leguminous plant *Sesbania rostrata* and the bacterium *Azorhizobium caulinodans* strain ORS571 is in many aspects a unique one (De Bruijn, 1989). In contrast to most legumes that form nitrogen-fixing nodules only on the roots, *S. rostrata* can be nodulated on the roots and the stem. The aerial nodules are formed at predetermined sites that are dormant root primordia located in vertical rows along the stem. These primordia develop into roots when immersed in water and/or into nodules upon infection with *A. caulinodans*. Root and stem nodule organogenesis are very similar and are intermediate between the indeterminate and determinate types of nodule development (Ndoye et al., 1994). Nodulation of *S. rostrata* stems is relatively unaffected by combined nitrogen and can be very extensive resulting in an extremely high fixation rate per plant. The closest relative of *A. caulinodans* is the diazotroph *Xanthobacter*, and *A. caulinodans* is unique amongst rhizobia in being capable of both symbiotic and free-living nitrogen fixation, the latter with assimilation of the formed ammonium for growth.

In this paper, we concentrate on the molecular analysis of the initial stages of this symbiosis. First, bacterial functions will be described. From ORS571, three nodulation (*nod*) loci were isolated. These loci are involved in the production of Nod factors that are signal molecules to the plant. To achieve a proper infection and nodule development, the bacteria need the proper cell surface structure. An operon containing genes involved in the synthesis of lipopolysaccharide (LPS) and absolutely required for nodulation, was isolated. Also the involvement in nodulation of an azorhizobial endoglucanase gene will be discussed. The differential display technique, a method based on polymerase chain reaction (PCR) amplification and visualization of mRNA populations, was used to isolate novel *S. rostrata* genes that are induced during bacterial infection. This will be described in the last part of the paper.

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The *nod* genes of *Azorhizobium caulinodans* ORS571

The nodulation capacity of rhizobia (*Azorhizobium*, *Bradyrhizobium* and *Rhizobium* species) is largely dependent on their *nod* genes. These genes are also the major genetic determinants of the bacterial host range (for a comprehensive overview, see Schultze et al., 1994a). Three *nod* loci were isolated from ORS571. These are located on the chromosome and are not closely linked to each other. One locus contains the regulatory gene *nodD* (Goethals et al., 1990), the second contains a large operon comprising the genes *nodABCSUIJZorf9* and possibly other genes (Goethals et al., 1989; Geelen et al., 1993, 1995a) and the last locus contains the gene *nolK* (Goethals et al., 1992a). Five of the six borders of these three loci (the border downstream of *orf9* has not yet been determined) are formed by genetic elements that are reiterated in the ORS571 genome and/or have homology to insertion elements. Moreover, the location of these elements corresponds to a shift in the GC content: the *nod* loci have a GC content of 47%-55% whereas sequences outside have a GC content similar to that of the ORS571 genome (67%) (Goethals et al., 1992a; Geelen et al., 1995b). Also the *nif* and *fix* genes have a GC content similar to the ORS571 genome. This suggests that *A. caulinodans* acquired its *nod* genes by horizontal gene transfer and that it evolved from a diazotrophic to a symbiotic bacterium. The *nolK* and *orf9* genes are so far unique to ORS571, but the other *nod* genes have homologous counterparts in (brady)rhizobia although the degree of conservation is very low.

Regulation of the *nod* genes

The common theme for *nod* gene regulation in rhizobia consists of the *nodD* gene product that belongs to the LysR family of transcriptional regulators, plant derived flavonoid signals, and the '*nod* box', located in the 5' region of the *nod* operons that are regulated by NodD. NodD binds to the *nod* box and activates the transcription of the downstream located genes in the presence of flavonoids. In many rhizobia, this regulatory circuit is extended with additional regulatory aspects such as multiple copies of the *nodD* gene, autoregulation of the *nodD* genes, the presence of a *nod* gene repressor and a two-component regulatory system acting synergistically with NodD activation (reviewed in Schultze et al., 1994a).

In the case of *A. caulinodans*, only evidence for the most elementary form of this regulatory system has been found. One *nodD* gene copy is present (Goethals et al., 1990), it is constitutively expressed (Geelen et al., 1995b) and shows no autoregulation. It is absolutely required for the expression of the *nodABCSUIJZorf9* and *nolK* loci and for nodulation (Goethals et al., 1990, 1992b). Transcription of these two loci is induced by NodD in the presence of the host plant-exuded flavanone liquiritigenin or the related flavanone naringenin (Messens et al., 1991).

In the promoter sequences of the *nodABCSUIJZorf9* operon and the *nolK* gene, *nod* box sequences were found with low degree of similarity to *nod* box elements of (brady)rhizobia.

From the comparison of the *nod* box sequences of (brady)rhizobia and *A. caulinodans* and site-specific mutation analysis, a modular build-up of the *nod* box was proposed (Goethals et al., 1992b). In this model, the *nod* box carries two (sometimes three) NodD-binding sites with the inverted repeat structure A-T-C-N_9-G-A-T. Binding of NodD to the *nod* box is enhanced by the inducer flavonoid, suggesting a direct interaction of NodD with the inducer (Goethals et al., 1992b).

Nod factors of ORS571 and their biosynthesis

Enzymes encoded by the *nod* genes are involved in the biosynthesis and secretion of the Nod factor signals. Upon perception by the host plant, Nod factors induce the nodule developmental program. When Nod factors are applied in a pure form to the roots of plants, they induce several effects that are related to nodulation (reviewed by Spaink and Lugtenberg, 1994). Moreover, Nod factors are determinants of the host specificity as the structural requirements are different for different hosts (Schultze et al., 1994a). The azorhizobial Nod factors are chitopentasaccharides (a minor part consists of tetrasaccharides) acylated at the non-reducing end with $C_{16:0}$, $C_{18:1}$ or $C_{18:0}$. This core structure can be modified with a N-methyl and O-carbamoyl group at the non-reducing end and a *O*-D-arabinosyl branch at the reducing end (Mergaert et al., 1993).

We studied the biochemical role of the proteins encoded by the different *nod* genes and the Nod factor biosynthesis pathway. The strategies used were (i) sequence comparison of Nod proteins with proteins of known function; (ii) *in vitro* synthesis of Nod metabolites using purified or partially purified proteins and defined substrates; (iii) analysis of Nod factor structures produced by *nod* gene mutants; and (iv) expression of *nod* genes in various combinations in *Escherichia coli* and analysis of Nod metabolite structures. From the results, the following conclusions were made. Nod factor synthesis starts with the NodC protein that is a *N*-acetyl-glucosaminyl transferase using UDP-*N*-acetylglucosamine as substrate (Geremia et al., 1994). The reaction products of NodC were chemically characterized as chitooligosaccharides with a polymerization degree from two to five (Mergaert et al., 1995). The NodB protein acts after NodC and removes the N-acetyl group at the non-reducing end of chitooligosaccharides (John et al., 1993) and the products of the NodC protein (Mergaert et al., 1995). The NodS protein, previously shown to be involved in Nod factor methylation (Geelen et al., 1993), transfers *in vitro* a methyl group from *S*-adenosyl-L-methionine to chitooligosaccharides, deacetylated at the non-reducing end (Geelen et al., 1995c). Chitooligosaccharides, N-acetylated or N-acylated at the non-reducing end are not used as substrate. This suggest that the NodS protein acts after the deacetylation step by NodB and before the acyl transfer, a finding that was confirmed with *in vivo* experiments (Mergaert et al., 1995). *E. coli* strains, expressing *nodABC* or *nodABCS*, produce lipochitooligosaccharides (Mergaert et al., 1995), showing that NodA is involved in acyl transfer, in agreement with results of others (Atkinson et al., 1994; Röhrig et al., 1994).

The NodU and NodZ proteins are involved in carbamoylation and arabinosylation of Nod factors, respectively (Geelen et al., 1995a). This was deduced from two lines of evidence.

First, NodU resembles a protein from *Nocardia lactamdurans* that is involved in the carbamoylation of an antibiotic and NodZ is the azorhizobial counterpart of the NodZ protein of *Bradyrhizobium japonicum* that is involved in fucosylation of Nod factors. The second evidence was obtained by analysis of Nod factors produced by strains mutated in the respective genes.

Secretion of Nod factors by *A. caulinodans* is in part mediated by the NodI and NodJ proteins (M. Fernández-López, unpublished results). These proteins belong to the family of ABC transporters that are involved in transport of molecules across membranes (Reizer et al., 1992). A mutant in the *nodJ* gene is severely affected in the secretion of Nod factors. Secretion can be restored by reintroduction of the *nodIJ* genes in the mutant whereas these genes have no influence on the Nod factor structure.

LPS of *A. caulinodans* are involved in nodulation of *S. rostrata*

The bacterial cell surface plays a role in nodule infection and uptake in the plant cell. It is believed that exopolysaccharides are important in the formation of indeterminate nodules. On the contrary, LPS are important for bacterial infection of determinate nodules. An ORS571 locus with a high degree of similarity to the *rfb* locus of *Salmonella typhymurium*, involved in *O*-antigen synthesis, was isolated (Goethals et al., 1994). The locus contains an operon that is involved in the synthesis of dTDP-L-rhamnose. A mutation in this locus had a pleiotropic effect including a rough colony morphology, non-motility, clumping in liquid medium and an increased cell surface hydrophobicity. The mutant proved to be defective in the synthesis of LPS as was shown with sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. The mutant was also impaired in nodule formation on the roots and the stem of *S. rostrata*. It formed no functional nodules, but instead slight swellings or pseudonodules, devoid of bacteria, were formed on the roots, a phenotype that resembled the effect of pure Nod factors (W. D'Haeze, unpublished results). Whether the nodulation deficiency is solely due to the altered LPS structure is not known. The other characteristics of the mutant might also contribute to some extent. Moreover, other surface polysaccharides might also be affected by the mutation.

An *A. caulinodans* endoglucanase gene

At several stages during infection, plant cell walls are crossed by the invading bacteria. Possibly, hydrolytic enzymes of plant and/or bacterial origin are degrading cell wall polymers to facilitate invasion. An endoglucanase gene (*egl*) from ORS571 was isolated and sequenced (Geelen et al., 1995d). The deduced protein sequence consisted of 1836 amino acids and showed the presence of several domains including a catalytic domain, two cellulose-binding domains and an 8-fold reiterated motif. The endoglucanase activity was expressed at low but constitutive levels and was not influenced by the *nod* gene regulatory system. A mutation in *egl* completely disrupted detectable endoglucanase activity. However, the mutant was not affected in nodulation on the stem and roots of *S. rostrata*. It is still possible that ORS571 *egl* has a minor role in symbiosis or that cryptic endoglucanase gene(s) are present. An alternative function for the endoglucanase, not yet investigated, could be the liberation of bacteria from senescent nodules. It is however unlikely that the protein serves in the generation of carbon sources from cellulose as ORS571 can not grow on glucose or cellobiose.

S. rostrata genes expressed during *A. caulinodans* infection

Since *S. rostrata* stem nodulation occurs synchronously at predetermined sites and nearly all dormant root primordia infected with ORS571 develop into nodules, this interaction provides a sensitive system to study plant gene expression during the early stages of nodule development. The differential display technique was used to compare gene expression in stem-located root primordia, uninfected or infected with ORS571 (Goormachtig et al., 1995). This resulted, hitherto, in the isolation of five cDNA clones (didi-2, didi-13, didi-20, didi-24, and didi-50) corresponding to novel genes with enhanced expression in the root primordia after ORS571 infection. In addition, a cDNA (didi-42) corresponding to the previously identified leghemoglobin gene *Srlb6*, was isolated. The temporal expression patterns of the cDNA clones was studied with northern analysis. Between one and five days after bacterial infection, mRNAs corresponding to the didi clones were induced and only for didi-20 a clear basal expression in uninfected primordia could be detected. Expression of the gene corresponding to didi-50 was too low to detect. For didi-2, didi-13, didi-20, and didi-24 it was also shown that its expression was depending on Nod factor signalling as an ORS571 *nodA* mutant was unable to induce the expression of the corresponding genes. DNA sequence similarity searches revealed several interesting findings. The didi-2 cDNA was similar to hydroxyproline-rich glycoproteins. The didi-13 and didi-24 clones resembled several class III plant chitinase genes. Didi-20 was similar to chalcone reductases and didi-50 to the soybean nodulin cDNA GmN#93 (Goormachtig et al., 1995).

With respect to the role in nodulation of the proteins encoded by some of these cDNAs, a number of speculations can be made. First, chitinases, enzymes from the phenylpropanoid pathway, such as chalcone reductase, or hydroxyproline-rich cell wall proteins all are implicated in plant responses to pathogens. In this respect, these enzymes could be part of a hypersensitive response to autoregulate nodulation (Vasse et al., 1993). Secondly, they could be involved in nodule development. Several proline-rich nodulins (proteins, induced during nodule infection or specifically found in nodules), such as ENOD2 or ENOD12, have been correlated with nodule morphogenesis or nodule invasion (Scheres et al., 1990; van de Wiel et al., 1990). Proteins of the phenylpropanoid pathway could control the production of certain flavonoid compounds acting as auxin transport inhibitors, thereby influencing hormone balances and nodule development (Yang et al., 1992). Finally, some of these proteins could control Nod factor activity. Chitinases can degrade Nod factors and thus control their quantity and biological activity (Schultze et al., 1994b; Staehelin et al., 1994). The enzymes of the phenylpropanoid pathway could be involved in the increased synthesis of *nod* gene-inducing flavonoids (Ini) (Recourt et al., 1991). The latter hypothesis seems very attractive as chalcone reductase is involved in the synthesis of liquiritigenin, the natural inducer for ORS571 *nod* genes.

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INTEGRATION OF PLANT AND BACTERIAL METABOLISM IN NITROGEN FIXING SYSTEMS

STREETER, J.G.
Dept. Horticulture and Crop Science, Ohio State University/OARDC, 1680
Madison Avenue, Wooster, OH 44691, USA

1. INTRODUCTION

Portions of this topic have been included in other recent reviews (McDermott et al. 1989; Day, Copeland 1991; Streeter 1991; Gordon 1992). In general, the literature cited here has been limited to papers not cited in those reviews. All of these earlier reviews contain schemes that illustrate processes of transport and metabolism in nodules; although these are very useful in providing a framework for understanding nodule function, they are not reproduced here because of space constraints. Because function is often related to the structure of nodules, the recent summary of nodule anatomy by Brown and Walsh (1994) is also noteworthy. In this report the structure of nodules from 43 species in 31 different genera of legumes is documented in a tabular summary of features and in a helpful selection of micrographs.

2. LEGUME NODULES - CARBON METABOLISM

Reviewers of this subject (like those cited above) generally conclude that C_4 dicarboxylic acids are the main carbon substrate for bacteroids. Most of the recent experimental evidence continues to support this view. Because most of the details of the biosynthetic pathways for asparagine and ureides have been established, there has been less recent effort on nitrogen metabolism and this area will not be covered in detail. The Vance group continues the molecular characterization of enzymes of N assimilation in nodules and this effort has recently been reviewed (Vance et al. 1994). One issue in N metabolism that remains unresolved is the importance of the γ-aminobutyrate shunt as a bypass for α-ketoglutarate dehydrogenase which is inhibited under the microaerobic conditions in bacteroids (Salminen, Streeter 1990). Although the shunt appears to be inactive in *Bradyrhizobium japonicum* (Salminen, Streeter 1990), recent

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evidence for *Rhizobium meliloti* bacteroids indicates that the shunt is an important mechanism for glutamate breakdown in these bacteroids (Fitzmaurice, O'Gara 1993).

2.1 Recent studies - enzymes

Incoming sucrose is the primary source of reductant in legume nodules, and starch may provide a significant temporary storage form of carbohydrate in nodules (Streeter 1991). There have been few studies of starch metabolism, but recent reports of α-glucosidase activity (Kinnback, Werner 1992) and starch synthase, α-amylase, and starch phosphorylase activities (Forrest et al. 1991) in soybean (*Glycine max*) nodules are consistent with a rapid turnover of starch. In the latter study, a comparison of enzyme profiles over time in Fix^+ and Fix^- nodules showed that N_2 fixation was correlated with the ability of nodules to rapidly break down starch.

Based on a comparison of enzyme levels in infected versus cortical tissues of soybean nodules, it appears that most of the sucrose breakdown occurs in the infected zone (Gordon 1991). Glycolytic activity within the infected zone is probably high in both infected and uninfected cells, based on a study of the localization of glyceraldehyde-3-phosphate dehydrogenase (Zammit et al. 1992). The importance of the pentose phosphate pathway in the breakdown of hexose appears to be greater in ureide-exporting nodules based on the activity patterns of the relevant enzymes in *Pisum sativum, Lupinus luteus, Vicia faba, Vigna unguiculata, Cajanus cajan,* and *Cicer arietinum* nodules (Hong, Copeland 1990). The Copeland group continues to document biochemical properties of the the glycolytic enzymes in host cytosol, with recent reports on the purification and properties of NAD-dependent glyceraldehyde-3-P dehydrogenase (Copeland, Zammit 1994), phosphofructokinase (Vella, Copeland 1993), and glucose-6-P dehydrogenase (Hong, Copeland 1991) from soybean nodules.

In bacteroids, malic enzyme has received most of the recent attention because, if 4-carbon acids are the main carbon source for bacteroids, this enzyme would play a key role in supplying the pyruvate needed to operate the TCA cycle. Both NAD- and NADP-dependent enzymes have been described and partially purified; these forms have very different Km values for malate, the NAD enzyme having a Km of about 2 mM and the NADP enzyme having a Km of about 0.1 mM (Kimura, Tajima 1989; Copeland et al. 1989; Trinchant, Rigaud 1990). Genetic analysis of TCA cycle enzymes has begun with the cloning of a fumarase gene from *B. japonicum*; because mutants retain some fumarase activity, a second enzyme is present in this organism (Acuña et al. 1991). In general, glycolytic enzyme activities are low and TCA cyle enzyme activities are high in bacteroids

(Day, Copeland 1991; Streeter 1991), and this is consistent with recent evidence for the repression of glycolytic enzymes by supplying succinate to several strains of *Rhizobium* and *Bradyrhizobium* (Mandal, Chakrabartty 1993). However, in the case of *R. tropici*, profiles of glycolytic and TCA cycle enzymes grown with glucose, glutamate, or malate when compared with profiles in bacteroids suggest that this organism could use either dicarboxylates or sugars in the nodule (Romanov et al. 1994).

2.2 Recent studies - ^{14}C labeling

This approach is potentially more valuble in elucidating overall nodule function because it integrates the activity of multiple enzymes and compartmentation within the nodule. Carbohydrate metabolism in *C. arietinum* nodules has been explored by injection of labeled glucose, fructose or sucrose and analysis of labeling patterns in the same sugars; the conclusion was that sucrose synthesis may occur in nodules, but the significance of this is not clear (Singh et al. 1994). Two groups have recently taken advantage of the high activity of PEP carboxylase in nodules to rapidly label intact *P. sativum* and *G. max* nodules with $^{14}CO_2$; following very short labeling periods, bacteroids were rapidly isolated and examined for patterns of labeling in amino acid and organic acid fractions (Rosendahl et al. 1990; Salminen, Streeter 1992). Both the patterns of label distribution and the kinetics of labeling of bacteroid pools indicate that malate is the major dicarboxylic acid supplied to bacteroids in these nodules. Rapid isolation of bacteroids following incubation of detached but intact *G. max* nodules with ^{14}C-sucrose was used to study the transfer of sucrose to bacteroids in this system (Streeter, Salminen 1993). Although labeled sucrose was found associated with the bacteroid fraction after incubations of 5 or 10 minutes, this was found to be due to the absorption of sucrose to the surface of bacteroids during isolation.

2.3 Recent studies - bacterial mutants

Previous reports indicate that mutants in carbohydrate metabolism generally give Fix$^+$ nodules whereas mutants defective in some step of the TCA cycle give Fix$^-$ nodules (Day, Copeland 1991; Streeter 1991). Recent reports of carbohydrate transport mutants of *R. meliloti* (Fix$^+$, Hornez et al. 1994), a succinate uptake mutant of *B. japonicum* (Fix$^-$, El-Din 1992) and an isocitrate dehydrogenase mutant of *R. meliloti* (Fix$^-$, McDermott, Kahn 1992) are consistent with earlier results. Among the more interesting mutants reported recently are mutants of *R. meliloti* lacking the NAD-dependent malic enzyme but retaining the NADP-dependent activity (Driscoll, Finan 1993). These mutants are Fix$^-$ thus providing strong support for the notion that 4-carbon dicarboxylic acids are the principal substrates for these bacteroids. Mutants of *R. meliloti* lacking gluconeogenic enzymes have also been studied by Finan's group and these mutants provide complex phenotypes as bacteroids. The overall conclusion from this work is that gluconeogenesis is probably not essential in the symbiotic state, but some metabolism of hexose may be required for full symbiotic competence (Finan et al. 1991).

2.4 Recent studies - other carbon substrates

Although it is not a substrate supplied by the host, the importance of poly-ß-hydroxybutyrate (PHB) as a metabolite in *B. japonicum* bacteroids has recently been suggested by the studies of Bergersen and Turner (1992, 1993). Their results show that, depending on O_2 concentration and the concentration of carbon substrate (e.g. malate) supplied to bacteroids, PHB may be accumulated or rapidly broken down. They propose that PHB may act as a "buffer" for the TCA cycle (see McDermott et al. 1989), alternately serving as a sink for or supply of pyruvate (Bergersen et al. 1991). The very high levels of ß-hydroxybutyrate dehydrogenase reported in *R. tropici* bacteroids (Romanov et al. 1994) is consistent with this notion. However, PHB appears to be unimportant in *R. meliloti* because mutants unable to synthesize PHB have normal nodulation and N_2 fixation activity (Povolo et al. 1994).

Proline is still considered by some to be an important carbon source for *B. japonicum* bacteroids based on the stimulation of acetylene reduction activity following supply of proline to nodulated roots (Zhu et al. 1992). The gene coding for the key enzyme of proline synthesis has been cloned from soybean nodules and the enzyme is mainly localized in the cytoplasm of infected cells (Delauney, Verma 1990; Szoke et al. 1992). That proline is not an important carbon source is indicated by the failure of proline to be transported into symbiosomes from soybean nodules (Udvardi et al. 1990) or into bacteroids from cowpea nodules (Glenn et al. 1991). Thus, the situation with respect to proline remains confusing and unresolved. It has been suggested that proline may be an important carbon source for bacteroids in environmentally stressed nodules (Kohl et al. 1994) and this is consistent with elevated levels of mRNA coding for pyrroline-5-carboxylate reductase in osmotically stressed soybean roots (Delauney, Verma 1990).

Space constraints prevent a thorough analysis of recent reports on other alternative carbon substrates for bacteroids and these possibilities can only be listed: sucrose on the basis of active uptake of sucrose by cultured *R. tropici* (Romanov, Martinez-Romero 1994); malonate, based on compartmentation of the acid and on other data for *L. luteus* nodules (Schramm 1992); oxalate, based on the oxalate concentration in *V. faba* nodules and the ability of this acid to support nitrogenase activity in isolated bacteroids (Trinchant et al. 1994); glucose, based on the labeling patterns in bacteroids from *L. luteus* nodules following feeding with specifically labeled ^{14}C-glucose (Fedulova et al. 1990).

Two conclusions seem appropriate. First, data supporting the importance of dicarboxylic acids is very extensive and convincing; other "candidates" for important carbon sources need to be supported by data from a wide variety of experimental approaches (see Streeter 1991). Second, we should remain open to the possiblity that carbon metabolism is not the same in all legume nodules. This possibility becomes increasingly plausible as we understand the large genetic differences among species of rhizobia.

3. LEGUMES - CARBON TRANSPORT

3.1 Genetics of dicarboxylate transport in rhizobia

Previous analyses of the *dct* genes in rhizobia also provide strong support for the notion that dicarboxylic acids are the principal form of reduced carbon supplied to bacteroids (Day, Copeland 1991; Streeter 1991). Regulatory circuits for the three *dct* genes are complex and the reader is referred to recent papers by Jording et al. (1992, 1994) for a detailed review. Recent studies on the *dctA* gene confirm its absolute essentiality for nitrogen fixation (vanSlooten et al. 1992) and predict the orientation of the DctA protein in the bacteroid cytoplasmic membrane (Jording, Pühler 1993). Whereas the expression of *dctA* is amplified by the availability of substrate (Jording et al. 1992), the expression of *dctB* is constitutive and the gene product has strong auto-phosphorylation activity (Giblin et al. 1995). The DctB protein controls the activity of DctD and mechanisms are being pursued by deletion analysis of *dctD* (Huala et al. 1992; Lee et al. 1994). A regulatory link between *dctB* and the expression of *nifHDK* in *R. meliloti* has also been suggested (Birkenhead et al. 1990). Previous confusing results showing the growth of Dct$^-$ mutants on aspartate have recently been clarified with the demonstration of a second mechanism for aspartate (and glutamate) transport in *R. meliloti* (Watson et al. 1993).

Since the first demonstration of the importance of the Dct system to nitrogen fixation, it has been of obvious interest to construct and test rhizobial strains having increased capacity of the uptake of dicarboxylic acids. Two reports of such tests have recently appeared. In the first, a *R.*

meliloti strain having 5-fold greater expression of *dctA* was used to form nodules on *Medicago sativa* plants. Plants had 30 to 60% greater acetylene reduction activity per plant, but there was no increase in plant growth (Rastogi et al. 1992). In the second report, a *R. meliloti* strain having additional copies of both *nifA* and *dctABD* was used to form nodules on *M. sativa* plants in a field study. Where soil organic matter was extremely low (0.9%) there was a positive effect on plant biomass but, at three other sites there was no significant effect of the "improved" strain on plant yield (Bosworth et al. 1994). These results have led to the reasonable suggestion that nitrogen fixation may limit the transport of dicarboxylates to bacteroids and not vice versa (Jording et al. 1994).

3.2 Transport across the symbiosome membrane (SBM)

This major control point for metabolite transport continues to receive attention. Recent reports have emphasized the selectivity of the SBM in *G. max* (OuYang et al. 1990) and in *L. luteus* (Tomaszewska et al. 1991). Namely, symbiosomes take up malate and succinate at rates far in excess of the rates for the transport of sugars or malonate or amino acids. Although the uptake of glutamate by pea nodule symbiosomes via an exchange mechanism has been proposed (Rosendahl et al. 1992), symbiosomes from other nodules transport glutamate poorly if at all (OuYang et al. 1990; see Day, Copeland 1991). In addition, the SBM appears to have no mechanism for the transport of ammonium (Udvardi, Day 1990) and this is consistent with previous results indicating passive transport of ammonia from bacteroids to host (see Streeter 1991).

The mechanism by which dicarboxylates are transported across the SBM is coming into focus. Analysis of host gene expression in nodules revealed the presence of a major 26-kD protein in the SBM several years ago and, more recently, this protein has been identified as a membrane-spanning channel protein (Miao et al. 1992; Miao, Verma 1993). Phosphorylation of this protein is correlated with malate transport (OuYang et al. 1991) and recent studies with the protein incorporated into artificial lipid bilayers indicates that the protein is, in fact, an anion channel (Weaver et al. 1994). It is possible that the nodulin 26 protein is the mechanism by which dicarboxylates are transported through the SBM.

3.3 The driving force for dicarboxylate transport

Studies by Australian workers using intact symbiosomes from soybean nodules have demonstrated ATP-dependent formation of of an electrical potential gradient over periods of a few minutes and the formation of a ΔpH over a period of hours

(Udvardi et al. 1991; OuYang, Day 1992). These results suggested (but did not prove) the presence of an ATP-dependent proton pump in the SBM. Recently, Dubrovo et al. (1992) have succeeded in preparing well-sealed SBM vesicles from *L. luteus* nodules and, using a proton-specific fluorescent probe, very short time periods and all of the correct controls, have demonstrated clearly the presence of ATP-dependent proton pumps in the SBM. The orientation of the pumps would result in H^+ transfer from host cytoplasm to the symbiosome space. The proton gradient (high $[H^+]$ inside) created by these pumps probably supplies the driving force for dicarboxylate transport across the SBM and represents the mechanism by which the host controls carbon supply to the bacteroids. Rhizobia absorb carboxylic acids via a H^+/dicarboxylate co-transport system (i.e. DctA) in the cytoplasmic membrane (Tremblay, Miller 1984; Bhandari, Nicholas 1985) so, although it has not been directly measured, it is possible to predict that the pH of the symbiosome space is low relative to the two adjacent cytoplasms. Treatments of nodules affecting ATP generation in mitochondria or bacteroids (e.g. oxygen supply) may perturb these H^+ gradients and have rapid and dramatic effects on nitrogenase activity (Streeter 1995).

4. NON-LEGUME ASSOCIATIONS

4.1 *Azospirillum*/wheat associations

Stimulation of the growth of grasses by inoculation with *Azospirillum* has been known for over a decade and attempts to explain the basis for this response continue. Some workers report major increases in yield in field studies although the basis for these increases does not appear to be increased N input (e.g. Caballero-Mellado et al. 1992). A recent suggestion is that nitrite formed in nitrate respiration by the *Azospirillum* in combination with auxin provides the growth stimulus (Bothe et al. 1992). In some studies, increases in N content of the plants (Stancheva, Dinev 1992) or incorporation of ^{15}N from $^{15}N_2$ (Christiansen-Weniger, vanVeen 1991) indicate that N_2 fixation in the bacterium does contribute to plant N nutrition. It may be possible to exploit results indicating that release of organic acids by roots is related to N_2 fixation activity or to employ NH_4^+-excreting strains of *Azospirillum* (Christiansen-Weniger et al. 1992). Recently, a *nifH-gusA* fusion in *A. brasilense* has been used to study the patterns of colonization on wheat roots (Vande Broek et al. 1993).

It has also been known for some time that exposure of wheat roots to very low concentrations of the synthetic auxin 2,4-D inhibits lateral root growth and results in the formation of nodule-like structures ("*para*-nodules"). Recent studies on

2,4-D application coupled with *Azospirillum* inoculation have demonstrated that these structures may provide a low [O_2] environment for the bacteria (Zeman et al. 1992; Christiansen-Weniger 1992). Although acetylene reduction rates are typically very low in these studies (Zeman et al. 1992), use of ^{15}N has demonstrated the transfer of fixed nitrogen to the plant (Christiansen-Weniger 1992; Yu et al. 1993). Because natural auxins like indoleacetic acid substitute only poorly for 2,4-D (Sriskandarajah et al. 1993), it is difficult to see how these systems can be exploited directly.

4.2 Acetobacter/sugar cane association

Although N_2 fixation activity associated with the roots of sugarcane plants has been known for many years, only recently has the microsymbiont been isolated and identified. The bacterium, *Acetobacter diazotrophicus*, shows optimum growth in culture with 10% sucrose and a pH of 5.5 (Gillis et al. 1989). Two recent studies using electron microscopy suggest localization of the bacterium either in the vascular apoplast (Dong et al. 1994) or within the xylem elements of the shoots (James et al. 1994). Because this interesting organism also produces indoleacetic acid, stimulation of sugarcane growth may be due to both hormone and nitrogen effects (Fuentes-Ramirez et al. 1993).

5. SUMMARY

In my view, the most significant developments in this subject area over the past few years are: (1) progress in understanding the regulation of the *dct* genes and the localization and operation of the gene products, (2) the failure of engineered strains with amplified DctA to improve (in general) N input or plant growth, (3) demonstration of an anion channel protein and an H^+-pumping ATPase in the SBM, components of a possible mechanism for the transport of dicarboxylic acids through this major barrier, (4) additional evidence (malic enzyme mutants, short-term labeling studies, etc.) supporting the concept that C_4 dicarboxylic acids are the principal source of reducing equivalents for bacteroids.

Although reports suggesting the importance of other carbon sources (oxalate, proline, glucose, malonate, etc.) are interesting, these need to be viewed against the hundreds of papers supporting the "C_4 dicarboxylate view". Questions that need to be answered before a particular carbon source can be considered as a likely source of reductant for bacteroids include: (1) Is the compound rapidly labeled to high specific activity in nodules following exposure of plants to $^{14}CO_2$?, (2) Are enzymes required for synthesis of the compound present with high activity in the host cytoplasm?, (3) Does the compound readily pass through the SBM?, (4) Is the compound absorbed under microaerobic conditions *in vitro* by purified bacteroids via an active mechanism?, (5) Are the enzymes required for catabolism of the compound present with high activity in bacteroids and is the labeled compound, when supplied to pure bacteroids, rapidly converted to $^{14}CO_2$?, (6) Does the compound, when supplied to bacteroids under appropriate conditions *in vitro*, support respiration and nitrogenase activity?, (7) Are bacterial mutants unable to transport or metabolize the compound Fix$^-$? Also, experimental tests of these questions should compare the compound of interest to malate or succinate in the appropriate situations.

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NITROGEN FIXATION IN AGRICULTURE: APPLICATION AND PERSPECTIVES

VANCE, C.P.[1] AND GRAHAM ,P.H.[2]
[1]USDA/ARS Departments of Agronomy and Plant Genetics, [2]Department of Soil, Water and Climate, University of Minnesota, St.Paul, MN 55108 USA

Individual protein consumption of the 5.3 billion people on Earth averages about 70 g of protein/day, or 23 million tons of nitrogen (N)/annum (Waggoner, 1994). To maintain this level of intake as the Earth's population doubles over the next 40 years, will necessitate a doubling or tripling of crop production, despite a significant deterioration of much prime agricultural land, and will require the utilization of large areas now considered marginal. Judicious managment of N in the environment will be essential . Nitrogen acquisition and assimilation is second only to photosynthesis in terms of importance for plant growth and development, yet N is perhaps the single most important factor currently limiting crop yields. The striking increase in cereal grain yields in developed countries between 1950 and 1990 was directly attributable to 10-fold increase in N fertilizer use (Waggoner, 1994). However, further increases are unlikely to be achieved by increased N fertilization. The increased use of N in developed countries has led to leaching of toxic NO_3^- into groundwater and volatilization of N oxides into the environment (Bohlool, 1992). In developing countries, the high cost of N fertilizer and the energy requirements for production will limit its use. Biological N_2 fixation (BNF) accounts for 65% of the N currently utilized in agriculture and will be increasingly important in future crop productivity especially for sustainable systems, small scale operations, and marginal land utilization. It is imperative that we not only understand the contribution of this process to various agricultural systems, but also appreciate current limitations to BNF under field conditions. In this paper, we have selected areas of research that are key to improving field crop levels of BNF and discuss research strategies for their resolution.

GENETIC IMPROVEMENT IN THE HOST

Most legume species studied to date have exhibited genetic variability in traits related to BNF, including nodule number and mass, seasonal accumumulation of N, %N derived from symbiosis, acetylene reduction activity (ARA), host restriction of nodulation, and enzyme function associated with N assimilation (Herridge et al. 1994). Single gene loci regulate nodule number, host strain compatibility, and sensitivity to nitrate. When properly managed, such variation is more than sufficient to improve current rates of BNF in the major legume species (Buttery et al., 1992; Herridge et al., 1994), to ensure enhanced yields under N-deficient conditions, and, in specified conditions to provide significant N for succeeding or companion crops.

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Layzell and Moloney (1994) have suggested that increases in BNF of up to 300% could be achieved through host plant selection and effective crop management.

BNF in legumes can be enhanced through either indirect selection for traits, such as biological yield and N concentration under low soil N conditions, or by direct selection using traits associated with BNF including nodule mass, xylem ureide content (Herridge et al. 1994), ARA (Rosa and Bliss, 1986; Heichel et al., 1989), and ^{15}N isotope enrichment (Heichel et al., 1989; Herridge et al., 1994). Breeding activities with soybean, common bean, and alfalfa typify differences in the balance of direct and indirect approaches used to date. Improved BNF has resulted from indirect selection for yield and protein content in soybeans in the USA. Comparisons of modern soybean lines with both ancestral cultivars and agronomically superior plant introductions show that enhanced N accumulation is characteristic of newer soybean genotypes (Cregan, Yaklick, 1986). Moreover, field studies of the soybean cultivars 'Lincoln', 'Shelby', and 'Williams' which represent serial enhancement of the 'Lincoln' line, show that selection for increased yield resulted in higher N concentration, total N, and N_2 fixed (Coale et al., 1985). Duc et al. (1988) have shown through evaluation of 21 faba bean genotypes selected for increased yield that the highest yielding genotypes also fixed the greatest amount of N_2. Improvement of U.S. soybean cultivars came through selection for high yielding varieties grown in well fertilized soil with little to no control of rhizobial strain interactions. This selection strategy has resulted in a troublesome ecological situation in which agronomically superior lines of soybean frequently are nodulated by native *Bradyrhizobium japonicum* strains that are inferior in N_2 fixation to those in inoculum (Buttery et al., 1992). Thus maximum yields of U.S. soybean cultivars are rarely achieved under symbiotic growth conditions because they seldom derive more than 60% of their N from symbiosis. By comparison, in Brazil, soybean selection has been conducted in the presence of highly effective *B.japonicum* without N fertilizer (Dobereiner, 1987) to result in cultivars that require no added N for maximum yield. Also a marked increase in the production of soybean in the Brazilian cerrado was achieved by selection of antibiotic resistant strains of *B.elkanii* (Rumjanek et al., 1993).

The limited BNF ability of most varieties of *Phaseolus vulgaris* (Graham, 1981) led Bliss and coworkers to apply the backcross inbred method of population development to the transfer of superior BNF ability from "Puebla 152" into otherwise agronomically acceptable bush bean cultivars (Rosas, Bliss, 1986; Bliss, 1993). Improvement in bean was initally based upon both ARA and ^{15}N analysis then in subsequent generations upon seed yield and N accumulation data for plants grown under N limited conditions.

Several different approaches have been used in attempts to enhance BNF by alfalfa. Teuber et al. (1984) selected within two unimproved alfalfa germplasms first by growth on low N and then by transfer to a high N environment. Selection at low N was to emphasize symbiotic effectiveness, whereas growth on high N reflected differences in N uptake and assimilation. This approach improved herbage N concentration and dry matter production under controlled conditions, but not in the field (Teuber, Phillips, 1988). A similar approach with the cultivar Moapa 69 enchaced total forage N and %N derived from symbiosis, but did not affect yield (Phillips, Teuber, 1992). Increased nodulation obtained in this study may have resulted from enhanced release of flavonoid *nod*-gene inducers (Kapulnik et al., 1987). A multiple trait recurrent selection program for alfalfa at the University of

Minnesota selected for ARA, nodule and root mass, and shoot dry weight (Barnes et al., 1984). Reproducible positive results were obtained in the glasshouse, but results were equivocal in field grown plants (Heichel et al., 1989). During the evaluation of this material, root nodule enzymes associated with N assimilation were also assayed (Groat et al., 1984). Phosphoenolpyruvate carboxylase (PEPC) and NADH-glutamate synthase (GOGAT) activity were highly correlated with BNF and shoot growth. Subsequently, alfalfa germplasms previously selected for nitrogenase activity and agronomic performance were selected for differences in PEPC and GOGAT activities (Jessen et al., 1987, 1988). Crosses between high enzyme selections of the experimental line MnP1-11 and three other germplasm sources showed increased N yield, dry matter accumulation, and %N derived from fixation. It is not yet clear whether these gains were due solely to selection or whether they resulted in part from release of inbreeding depression in this outcrossing tetraploid species. Interestingly, plants selected for reduced PEPC and GOGAT activity showed decreased yield and N accumulation (Jessen et al., 1988) suggesting that threshold levels of these enzymes are necessary for normal growth.

Although few breeding lines can lay claim to having been selected directly for improved N_2 fixation, it is imperative that selection for this trait play an important role in the cultivars of the future. **Plant improvement strategies must ensure that new varieties fix as much N_2 as the best N_2-fixing germplasm currently available. This can be achieved in both developed and developing countries using established methods of direct and indirect selection. Improvement of N2 fixation and %N derived from symbiosis will depend upon recurrent selection programs that pyramid genetic traits desirable for this process including: an adequate balance of nodule mass and number; duration of activity; improved N assimilation; nitrate tolerance; and superior strain selection. Other important activities will be the introgression of supernodulation into agronomically acceptable cultivars, and the development of marker assisted protocols to select for improved BNF. Moreover, it will be increasingly important to obtain accurate quantification of N_2 fixed by trees, and select for improved yield and BNF in these species.**

STRAIN COMPETITION AND PERSISTENCE

When legumes are planted into soils containing indigenous rhizobia, the numerical superiority and more uniform distribution of the resident organisms limits nodule occupancy by the inoculant strain. This is usually attributed to competition for nodulation sites. To overcome this, Streeter's (1994) review of this phenomenon notes four approaches: strain selection for superior competitive ability; use of higher than normal inoculation rates; development of alternate inoculant and delivery systems; and studies on the genetic basis for strain competitiveness. To date these approaches have failed to consistently enhance inoculant strain response in the field. Inoculant strains have been shown to contribute significantly to crown nodulation but to produce few nodules on other parts of the root system (McDermott, Graham, 1989; Wadisirisuk et al., 1989). McDermott and Graham (1989) and Caetano-Anolles et al. (1992) attributed this to the inability of the inoculant strain to keep pace with the developing root system, and have identified inoculant strain mobility, rather than strain competitiveness, as the major factor limiting nodule occupancy. Thus, once crown nodules begin to senesce, the plant becomes dependent on nodules formed by rhizobia which may be significantly less efficient in N_2 fixation. Using mixtures of strains distributed uniformly throughout the test soil, significant differences in "strain competitiveness" are demonstrated, but Graham and McDermott (1989) suggest that rhizobia in soil and rhizosphere are not really in direct competition but must each complete infection events within a limited time period, with inefficient strains producing fewer nodules and appearing to be weak competitors. This hypothesis is consistent with the correlation between speed of nodulation and competitive ability (Stephens and Cooper, 1988; McDermott and Graham, 1990). While the selection of "competitive" strains is relatively straightforward, overcoming the problem of limited inoculant mobility in the soil is not. Four approaches to overcoming this limitation merit attention: **1) the identification of specific preferences or restrictions in nodulation; 2) the development of alternate inoculation procedures aimed at nodulation of lateral roots; 3) the use of fungicide or bactericide treated seed and inoculant strains resistant to these chemicals; and 4) improved duration and efficiency of N_2 fixation in crown nodules.**

Specificity in nodule formation occurs at all stages of the infection process, with early examples of plant cultivars that restrict nodulation by particular strains, or prefer to nodulate with one strain and not another often associated with plant introductions, and considered a problem affecting strain selection. Examples have included the pea cultivar Afghanistan in Europe (Lie, 1971) and the soybean cultivar Bossier in Nigeria (Pulver et al., 1982). Most such specificities appear to be elegant adaptations of Flor's (1947) "gene-for-gene" concept. Cregan and Keyser (1986 et *seq*) sought host restriction in nodulation as a means to overcome the nodule dominance of indigenous rhizobia belonging *to B.japonicum* serogroup 123 in Midwestern soils, and identified PI371607 and PI377758 as lines in which a high proportion of the nodules formed were due to the inoculant strain. The initial expectation was that all serogroup 123 strains would be excluded; further evaluation identified subgroups within serogroup 123, and showed these to differ in degree of restriction (Sadowsky et al., 1990). A single dominant gene identified in *B.japonicum* strain USDA 110 could complement the restriction nodulation phenotype of serogroup 123 (Sadowsky et al. 1991). A similar system controlling nodulation between primitive pea cultivars and the *R.leguminosarum* bv.*viciae* Tom (Fobert et al., 1991) is being evaluated for use in the strain specific nodulation of peas. **Continuing efforts must define these responses and determine the fundamental basis of restriction specificities.** In the search for host-strain pairs for use in host preference or restriction studies, *Rhizobium tropici* UMR1899 might well serve as a model strain. It is highly effective in symbiosis, but belongs to a species which is relatively uncommon in all but the more acid soils (Giller et al., 1994; Hungria et al., 1993; Graham et al., 1994).

While inoculant rhizobia are active in crown nodulation, such nodules have declined significantly in activity by 65 days after planting (DAP; Bergersen, 1958). McDermott and Graham (1989) showed that crown nodules accounted for essentially 100% of plant ARA 20 days after planting, but for less than 20% of the ARA by 76 DAP. Moreover, the maximum amount of N_2 fixed occurs after R_1 in soybean (Hardarson, 1993). An effective remedy to the developmental and spatial separation of infectible root region and inoculant strain may be the application of inoculum in liquid or band-applied formulations at times other than planting (Hardarson, 1993; Streeter, 1994). *Bradyrhizobium* application in irrigation water permitted inoculation rates of more than 1.3×10^{11} soybean bacteria per m^2 and resulted in better nodulation and yield as compared to seed applied rhizobia in heavy soils having few indigenous rhizobia

(Ciafardini, Barbieri, 1987; Ciafardini, Lombardo, 1991). Similar experiments are needed for soils having native rhizobia.

While strain "competitiveness" has been extensively studied, the ability of inoculant strains to persist in soil has been relatively ignored. Several studies demonstrate the continued existence of the inoculant strain in soil after periods of 6-12 years (Diatloff, 1977; Brunel et al., 1988). They are outnumbered, however, by studies (e.g., Brockwell et al., 1982) which show an inoculant strain essentially disappears within two to three years. There are many situations in which differences in strain persistence are difficult to explain. **We need to understand how factors such as ability to utilize specific substrates, strain differences affecting predation, and the importance of microniches in the soil affect saprophytic competence of siol rhizobia. Along the same lines, we need to determine what factors regulate the frequency of certain sym-plasmids in soil rhizobia and establish the origins of the many non-infective rhizobia in soil (Segovia et al., 1991) and the degree to which populations of these organisms vary from one soil to another.**

At first glance, the production of inoculants for any legume should pose few problems. Yet the process of inoculant production, though technologically simple and a showcase for our profession, has not had the success it might have, particulerly in developing countries. Olsen et al. (1994) describe inoculant production in Canada. Theirs is an exceptional, well-regulated program. Similar programs exist in a number of other countries (i.e., Australia, Brazil, South Africa, and Uruguay) that have a central laboratory responsible for the evaluation of inoculant quality, routine inoculant strain maintenance and distribution, regional trials for the evaluation of potential inoculant strains or strains for new legumes, and procedures for the establishment of standards and modification to inoculant strain recommendations (Thompson, 1992). By contrast inoculant production in many parts of the world leaves much to be desired, with the presence of suitable rhizobia in the inoculant entirely coincidental. **In most cases this is not a question of research priority, but one of training, of giving credit within the research establishment for practically-oriented field testing, and of appropriate farmer education.**

EDAPHIC STRESSES

The expansion of agriculture into areas of more marginal production exposes legume crops to additional edaphic stresses. Examples include the low soil pH in Brazil and drought across large areas of sub-Saharan Africa. Even currently-productive land is threatened by salinization and acidification. Using soil acidity as an example of approaches to questions of stress, we know that soil acidity affects both *Rhizobium* and legume host, involving aspects as different as survival and multiplication of the rhizobia on seed or in soil to the initial steps of infection. These effects can be due to hydrogen ion concentration per se, to aluminum and manganese toxicities, or the induced deficiency of calcium, molybdenum, and phosphorus. The relative importance of each factor will differ from soil to soil. Approaches to restifying soil acidity problems have included liming soil, pelleting seed with calcium carbonate or rock phosphate to provide a more favorable microclimate for initial infection, and selecting plant cultivars and rhizobia for tolerance to acid soil factors.

Liming is effective in intensive agricultural production systems but is expensive for low resource farmers in the tropics (Adams, 1978). Futher, soil amendments are usually applied only to the plough layer, encouraging shallow root growth and leaving plants vulnerable to drought (Spehar, 1995). In *R.meliloti*, the Ca levels needed for growth at acid pH may be 20-100 fold those required at pH 7 (Howieson et al., 1992). The interaction between calcium and pH is further evidenced by the fact that when EGTA was placed on lucerne roots growing at pH 5.2, the incidence of nodulation was reduced from 87% to 32% (Pijnenberg et al., 1990). Spehar (1995) suggests that the development of calcium efficient varieties is a major factor in soybean production in the Brazilian cerrado.

The pelleting of seeds to protect rhizobia sown into acid soil is a long-established practice (Brockwell, 1962), often misapplied (Thompson et al., 1975). Few studies of seed pelleting have considered persistence of the introduced strain beyond the first year. Howieson and Ewing (1986) found acid tolerance an important factor in the persistence of *R.meliloti* in soils of acid pH, but other studies report persistence of non-tolerant strains in soil, presumably in microniches of lesser acidity (Richardson and Simpson, 1989).

Rhizobia vary significantly in acid tolerance. Some isolates grow even better in somewhat acid culture medium (Date and Halliday, 1979), but many alfalfa rhizobia do not grow below pH 5.6 (Howieson et al., 1988). 73% of the cowpea rhizobia and 62% of the soybean isolates evaluated by Keyser and Munns (1979) grew at pH 4.5, and some strains of *Rhizobium tropici* grow to pH 4.0 (Graham et al., 1994). Various mechanisms have been proposed to explain acid pH tolerance (Glenn, Dilworth, 1994). Much may be learned from the adaptive acid tolerance response in *Salmonella* (Foster, 1992). Acid pH tolerance in rhizobia enhances nodulation and competitive ability in acid soils (Graham et al., 1982; Vargas and Graham, 1988). Parallel differences in host tolerance have been shown (Howieson and Ewing, 1986; Graham, 1988), with striking results when both host and rhizobia are acid tolerant (Ewing et al., 1989; Spehar, 1994 a,b). Cultivars of *Phaseolus vulgaris* nodulate reasonably at acid pH when either of the two symbionts is acid tolerant (Vargas, Graham, 1988), suggesting compensation in the molecular signalling between the two symbionts.

There are many questions concerning stress tolerance which need to be resolved. **What is the basis(es) for increased calcium requirement among rhizobia grown at acid pH? Can strain and cultivar selection for efficiency in calcium utilization be achieved in most symbioses? Studies are also needed to determine the basis of pH tolerance in rhizobia, and to relate this to the ecology of these organisms in the soil, and to their field performance under acid soil conditions. Further effort is required to understand how acid pH affects communication between host and rhizobia, and how this is modified when either or both of the symbionts are pH tolerant. Finally, the identification of other legume/Rhizobium acid and aluminum tolerant combinations must be a priority if significant development of the acid soils of the world is to be achieved.**

BIOLOGICALLY FIXED FOR CROPPING SYSTEMS

Species capable of BNF have played an integral role in cropping systems since the domestication of plants. Peoples and Craswell (1992) noted that N_2 fixing species have been prominently featured in rotations and intercropping, as alley crops in the inter-row space of tree plantations, as green manures, in agroforestry, and pasture systems. Mixed cropping with N_2 fixing species was the accepted norm prior to the

Haber Bosch process (Fujita et al., 1992). Over the last 50 years, the practical use of BNF technology has lost favor due to various economic, social, and political forces (Heichel, 1987). With the advent of increased population pressure, farming of marginal land, and degradation of the environment, research on N_2 fixation must include renewed efforts to deliver BNF technology to cropping systems. **Research and extension must convincingly demonstrate the economic and environmental benefits accrued from incorporating BNF into farm management, identify the best species to incorporate into cropping schemes, know the amount of N fixed by these species, and accurately predict the availability of fixed N as replacement for fertilizer N within intercrops and in rotations.**

In intercropping systems, species capable of BNF are grown interspersed with or between rows of non-N_2 fixing species to improve N nutrition and performance. However, there is not only a question of now much N is transferred, but often whether N transfer occurs at all (Ledgard, Steele, 1992; Peoples, Craswell, 1992). Evidence supporting the transfer of N from N_2 fixing crops to companion species is predominantly indirect (Heichel, 1987). Using ^{15}N, the amount of companion crop N derived from the N_2 fixing partner can reach up to 70% (Broadbent et al., 1982; Brophy et al., 1987), but usually approaches 20% or less (Fujita et al., 1992; Peples and Craswell, 1992) has been 0% (Ledgard et al., 1985; VanKessel, Roskoski, 1988). N transfer is a function of species examined and environment (Heichel, 1987; Ladha et al., 1992; Peoples, Craswell, 1992). N transfer experiments are most reliable when the species examined have similar growth habits and rooting patterns. Consistently the greatest amount of N transfer occurs in low N soils (Brophy et al., 1987; Russelle et al., 1994). In high N soils the N_2 fixing species is less competitive than the companion crop and N_2 fixation is decreased resulting in less N transfer. This observation underscores the need to develop N_2 fixing species that continue to fix N_2 in the presence of soil N. Another confounding feature of N transfer studies is that bidirectional transfer can occur (Tomm et al., 1994) resulting in futher errors in estimation.

Green manuring (GM) brings to mind the incorporation of *Astragalus* and *Azolla* residues into rice cultivation (Giller and Wilson, 1991) and the plow-down of forage-legume autumn growth for small grains production (Heichel, 1987). However, the discovery of stem nodule N_2 fixation by fast growing *Sesbania* and *Aeschyonomene* in water-logged conditions has provided a new alternative for GM in tropical environments with high cropping intensities (Ladha et al., 1992; Giller, Wilson, 1991). These species have phenomenal growth rates and can fix N_2 at rates equivalent to 10 kg N $ha^{-1}.day^{-1}$ (Alazard, Becker, 1987). When incorporated, the recovery of N by rice from *Sesbania* and *Aeschyonomene* is greater than that from fertilizer N (Ladha et al., 1992). This discovery raises the question of whether there are other species that fix large amounts of N_2 which might be incorporated into GM systems.

The agronomic approach to addressing the N replacement value of BNF addition to a cropping system has traditionally been to assess the yield of the succeeding non-N_2 fixing crop in the rotation as compared to its yield obtained from adding various rates of N fertilizer. This approach is problematical and requires several assumptions. Heichel (1987) reported the shortcomings of this methodology: 1) it does not distinquish BNF from soil derived N; 2) it does not reflect the fact that factors other than BNF can contribute to the yield response of the succeeding crop; 3) it does not take into account differences in availability of fertilizer N and symbiotic N; and 4) the amount of N actually incorporated into the system which is derived from the athmosphere is seldom measured. Thus, the net contribution of BNF to the succeding crop is frequently overestimated. These deficiencies can be resolved by using ^{15}N and/or a non-N_2 fixing legume (or other) reference crop. For the sake of efficiency and cost, all replacement studies should use, when available, a near isogenic non-N_2 fixing reference. These have become available for many species in the last 10 years (Vance, 1990).

CONCLUSIONS

A recent report (NSF 1994) concluded that "the outstanding global potential of BNF in agriculture and forestry will be realized only with the long term investment of significant funds". While that may be true, those funds are only likely to be forthcoming when we do a better job of relating fundamental research to applied aspects of BNF, and of extending the results obtained in the laboratory or field to on-farm conditions. Our sense in the American Midwest is that many farmers have forgotten N_2 fixation. We need to reinvigorate the issue, but at the same time to balance this with a continued focus on the third world, and how BNF could be used to reduce its food and nutrition problems. In this paper we have discussed those areas of BNF research which in our eyes could give the best response to research investment. While our views are, of course, subjective and open to question, we hope they will serve as a discussion point and as a spark to help better define some of these issues.

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SECTION 2

CHEMISTRY AND BIOCHEMISTRY OF NITROGENASES

NITROGENASE - AFTER THE STRUCTURE.

SMITH, B.E.
Nitrogen Fixation Laboratory, University of Sussex, Brighton, BN1 9RQ, U.K.

The publication, in 1992, of the 3-dimensional X-ray structures of the Fe and MoFe proteins of *Azotobacter vinelandii* nitrogenase represented a major step forward in our understanding of this enzyme. These structures and their subsequent refinement are described elsewhere in this volume (Smith et al., 1995) and herald a new era in nitrogenase biochemistry and chemistry. We are now in a position to pose mechanistic questions on a much more informed basis, *e.g.*:

(a) Where do substrates bind - at Fe or Mo or S or some combination of these? Precedents for N_2 being reduced at Fe or Mo sites exist and C_2H_2 can be reduced on S sites. Furthermore, there are many models in which N_2 is bound in a bridging mode between two metal sites. This type of complex can sometimes be reduced to form ammonia, although more frequently hydrazine is formed. There is little information on the reduction of nitrogenase substrates at sites in metal-sulphur cluster complexes. Since there is very strong evidence that the FeMoco cluster acts as the substrate binding and reducing site in nitrogenase, then it will be necessary to develop an understanding of nitrogenase substrate interactions with metal-sulphur clusters in order to attempt to understand the enzyme mechanism.

(b) How is MgATP hydrolysis coupled to electron transfer? Nucleotide binding to each protein has been observed (Eady et al., 1995). Furthermore, MgATP hydrolysis only occurs when both proteins are present. The crystal structure of Av2 (Georgiadis et al., 1992) shows half-site occupancy by an ADP molecule. This provides very good evidence for the binding site of nucleotides to the Fe protein and yet this site is a considerable distance from the surface of the protein and cannot therefore be regarded as a possible bridging site where a nucleotide binds both to the Fe and the MoFe protein. Is it possible that more than one type of site is involved during turnover? For example, the crystallographically observed nucleotide binding site in the Fe protein could be important for modifying the interaction of the Fe protein with the MoFe protein, but another site on the MoFe protein could be involved in other stages of the substrate reduction process and might be associated with a nucleotide bridge between

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the two proteins. If there were two distinct sites on nitrogenase for nucleotide binding, and hydrolysis occurred at both sites, then one puzzling feature of nitrogenase function, *viz.* that two ATP molecules are hydrolysed at every electron transferred, may be explained.

(c) What is the role of the P clusters? Are they directly on the electron transfer path from the Fe protein to FeMoco or do they act as suppliers of electrons only at critical points during turnover? There is now developing evidence (Kim, Rees, 1992; Thorneley et al., 1993; Peters et al., 1994) for the interaction surfaces between the Fe protein and the MoFe protein which indicates that the P clusters could be on the electron transfer path from the Fe protein to the FeMoco centres. However, there is no evidence available in the literature to indicate that the P clusters become further reduced from their dithionite-reduced form at any point during turnover. Thus there is no evidence that electrons always pass from the Fe protein 4Fe4S cluster to the P clusters. However there is evidence that the P clusters can become oxidized releasing electrons at a critical point during turnover when reducing the natural substrate N_2 or protons. This oxidation appears not to take place in the presence of carbon monoxide or of acetylene (Lowe et al., 1993). These observations of course do not exclude the possibility that the P clusters are involved in passing all electrons to FeMoco. However this hypothesis appears unnecessary on current data.

The above questions are essentially biochemical but the answers will be partially dependent on chemical experiments with defined complexes. These can identify possible enzymic mechanisms and suggest biochemical experiments to distinguish them. Probably the most interesting question is "Why has nature devised two such unusual metal-sulphur clusters to carry out nitrogen fixation?" If models of these clusters can be synthesised perhaps their properties will give us some clues.

It is clear that we can now generate more informed hypotheses concerning the action of nitrogenase. A great deal of experimental testing of these hypotheses is required, some of which is presented in the ensuing papers in this session.

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PROBING CATALYTIC FUNCTION THROUGH AMINO-ACID SUBSTITUTIONS IN *AZOTOBACTER VINELANDII* MOLYBDENUM-DEPENDENT NITROGENASE

NEWTON, W.E., FISHER, K., KIM, C.-H., SHEN, J.,
CANTWELL, J.S., THRASHER, K.S., AND DEAN, D.R.
Department of Biochemistry and Anaerobic
Microbiology, Virginia Polytechnic Institute and
State University, Blacksburg, VA 24061-0308, USA

As part of our studies to elucidate the functions of both types of prosthetic group in the MoFe protein of molybdenum-dependent nitrogenase (Mo-nitrogenase), we are attempting to map the catalytic surface of both the FeMo-cofactor (FeMoco) and the P-cluster pair by individually substituting amino-acid residues within their polypeptide environments and analyzing the catalytic, redox and spectroscopic consequences for the resulting altered MoFe proteins (Newton, Dean, 1993). There is substantial evidence that implicates FeMoco as the site of substrate reduction (Shah, Brill, 1977; Hawkes et al., 1984; Scott et al., 1990, Kim et al., 1995), which occurs through a series of events that starts with the sequential association and dissociation of the two nitrogenase component proteins (Hageman, Burris, 1978; Lowe, Thorneley, 1984). During this first stage, two molecules of MgATP are hydrolyzed and a single electron is transferred from the Fe-protein component (a homodimer of ca. 63kDa, encoded by *nifH*) to the MoFe-protein component (an $\alpha_2\beta_2$ tetramer of ca. 230kDa, encoded by *nifDK*). The transferred electron is generally believed to be accepted by the P-cluster pair before intramolecular transfer to first FeMoco and then bound substrate (for a review, see Newton, 1993). The native MoFe protein exhibits a S=3/2 electron paramagnetic resonance (EPR) signal that is unique among metallobiomolecules. This EPR signal arises in FeMoco.

Recently, the complete structure of both component proteins from *Azotobacter vinelandii* has been solved by x-ray crystallographic methods (Georgiadis et al., 1992; Kim, Rees, 1992a,b; Chan et al., 1993; Kim et al., 1993; for recent reviews, see Kim, Rees, 1994; Dean et al., 1993). For the MoFe protein, one FeMoco is ensconced within each α-subunit and is directly ligated by residues αCys-275 and αHis-442 only. Putative hydrogen-bonding interactions, however, occur among: (i) αGln-191 and αGln-440 and FeMoco's

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homocitrate moiety (one at each terminal carboxylate); (ii) αHis-195 and a central bridging sulfide; (iii) αArg-277 through a water molecule to αHis-195 and then to the central bridging sulfide; (iv) αArg-359, which lies across one of FeMoco's three central faces, with a sulfide of the [$MoFe_3S_3$] sub-cluster; and (v) αArg-96, which lies across a second FeMoco face, with both a sulfide in the [$MoFe_3s_3$] sub-cluster (through its ϵ-nitrogen) and a central bridging sulfide (through a terminal amino nitrogen). The third of FeMoco's central faces is occupied by αPhe-381. Each P-cluster pair bridges an α,ß subunit interface and consists of two [Fe_4S_4] sub-clusters triply bridged to each other by a disulfide bond and the γ-S of two cysteinyl residues, αCys-88 and ßCys-95. One [Fe_4S_4] sub-cluster is terminally ligated by αCys-62 and αCys-154, whereas the other is ligated by ßCys-70 and ßCys-153. ßSer-188 ligates the same sulfide as does ßCys-153, which may be the reason why altered MoFe proteins with substitutions at ßCys-153 retain nitrogen-fixing activity.

In this report, we compare with wild type the phenotypic and catalytic characteristics of altered MoFe proteins from *A. vinelandii* mutants, each having a single amino-acid substitution at a targeted residue, and use spectroscopic methods to confirm the extent and site of any detectable change. Mutant strains were constructed, grown and derepressed, extracts prepared, and proteins purified as previously reported (Brigle et al., 1987; Jacobson et al., 1989; Kim et al., 1995).

SUBSTITUTIONS IN THE FeMoco ENVIRONMENT.

Substitution at αHis-195 by either Gln or Asn to give the αH195Q- and αH195N-MoFe proteins, respectively, results in loss of the ability to reduce N_2 for both, but otherwise produces quite dissimilar phenotypes (Kim et al., 1995). The αH195Q-MoFe protein has almost wild-type levels of H_2 evolution (Burns, Bulen, 1965) and, like wild type, reduces C_2H_2 to C_2H_4 only (Dilworth, 1966). Both reactions are potently inhibited by N_2, even though it is not a substrate, resulting in the uncoupling of MgATP hydrolysis from substrate reduction without altering the rate of MgATP hydrolysis. This inhibition by N_2 of both H_2 evolution and C_2H_2 reduction catalyzed by the αH195Q-MoFe protein suggests occupancy of a common active site. In contrast, the αH195N-MoFe protein has ca. 30% of the wild-type H_2-evolution rate and produces both C_2H_4 and C_2H_6 from C_2H_2. Neither of these reactions is inhibited by N_2. A similar phenotype to the latter is also observed with the αQ191K-MoFe protein (Scott et al., 1990; 1992), but here an additional effect of the substitution is to invoke CO-sensitive H_2 evolution.

We sought to gain more insight into these C_2H_2-reduction reactions through attempts to correlate C_2H_6 production with the stereospecificity of C_2H_2 reduction by using C_2D_2 as substrate. Wild-type nitrogenase produces all or mainly *cis*-$C_2D_2H_2$ (Dilworth, 1966; Lin-Vien D et al., 1989). In separate high-flux (Fe:MoFe of 40:1) experiments under either 10% C_2H_2 or 4.5% C_2D_2, wild type produced no C_2H_6 but 4.2% of the total C_2H_4 was in the form of the *trans* isomer (determined at 988 cm^{-1} by FT-IR of the gaseous atmosphere). Similarly, the αH195Q-MoFe protein produced no C_2H_6 and only 1.1% *trans*-C_2H_4. Although the αQ191K-MoFe protein gave 10.6% of the total hydrocarbon product as C_2H_6, only 4.5% of the total ethylene was in the form of the *trans* isomer, i.e., the same as wild type. If C_2H_6 is formed on the same mechanistic pathway as C_2H_4 through a longer residency time on the enzyme and involves a reversible, enzyme-bound E-C_2H_5 intermediate, it might have been anticipated that those nitrogenases which produce C_2H_6 would have lost the stereospecificity of proton addition at this intermediate stage. Our results, however, suggest no such direct correlation exists for these altered enzymes.

The αH195Q-MoFe protein also exhibits hypersensitivity of C_2H_2 reduction to inhibition by CO (Kim et al., 1995); the K^{i} of 0.004% is ca. eight-times less than that for wild type (Hoch et al., 1960; Bulen et al., 1965). Because CO has been reported to relieve completely the inhibition by CN^- of total electron flux, thereby implying a common binding site (Li et al., 1982), we surmised that the αH195Q-MoFe protein would likely have increased sensitivity to CN^-. To test this hypothesis, we used high-flux experiments to investigate the effects of buffered NaCN solutions, with and without CO, on both the wild-type and αH195Q-MoFe proteins. As found previously, with 5 mM NaCN, the wild type produced CH_4 and NH_3 from the substrate, HCN, but suffered ca. 50% inhibition by CN^- of electron flux (2300 vs. 5000 electrons per reaction) compared to reactions without NaCN. In addition, the ratio of MgATP hydrolyzed per electron pair transferred to substrate (ATP/$2e^-$) about doubled (4.8 vs. 10.3). These effects were completely reversed by 10% CO when all flux resulted in H_2 evolution. Quite surprisingly with the αH195Q-MoFe protein, there was no inhibition of electron flux (4900 vs. 5000 e^-/reaction) with 5 mM NaCN, resulting in an approximate doubling of the CH_4 and NH_3 produced by wild-type. The ATP/$2e^-$ ratio remained constant at 5.0. The addition of 10% CO eliminated CH_4 and NH_3 production as expected but otherwise was without effect.

Computer modelling indicates that the putative hydrogen bond between the imidazole ϵ-nitrogen of αHis-195 and the central bridging sulfide of FeMoco could be replicated by an amino-N in the αH195Q-MoFe protein, thereby maintaining a structural feature critical for function. However, substitution by Gln at the αHis-195 position does have the major effects of: (i) eliminating N_2 reduction, but not N_2 binding; and (ii) eliminating the inhibitory effect, and therefore the binding of CN^-. These data suggest that both N_2 and CN^- have binding sites on the central face of FeMoco, which is involved with αHis-195, and that CN^- and CO do not share exactly the same binding site. Because N_2 binding inhibits C_2H_2 and H^+ reduction and because the affinity for CO is increased in the αH195Q-MoFe protein, it is likely that these molecules also have close-by binding sites that may overlap. Moreover, for each of these three substrates, there may well be more than one binding site. In fact, the altered MoFe protein with αArg-277 substituted by His shows inhibitor(CO)-induced cooperativity ($n = 2$) during C_2H_2 reduction, indicating at least three simultaneously-occupiable binding sites on the MoFe protein with one of the C_2H_2-binding site at the "Fe end" of FeMoco. Furthermore, altered MoFe proteins, which result from substitution by Lys at residues that interact with the "Mo end" of FeMoco, e.g., αArg-359 and αArg-96 (both of which hydrogen-bond with different sulfides in the $[MoFe_3S_3]$ sub-cluster), suffer CO-sensitive H_2 evolution, but only by ca. 50%. However, these substitutions have no effect on the K_m's for N_2 and C_2H_2. Substitution of αGln-191, which hydrogen bonds with the homocitrate at the "Mo end", by Lys also introduces CO-sensitive H_2 evolution, but additionally, causes the ability to fix N_2 to be lost. Thus, two H_2-evolution sites are indicated with one likely located on the $[MoFe_3S_3]$ sub-cluster.

SUBSTITUTIONS IN THE P-CLUSTER PAIR ENVIRONMENT.

The obvious structural symmetry surrounding each P-cluster pair as illustrated in the reported structural models (Kim, Rees, 1992a,b; Bolin et al., 1993) is not reflected by symmetry in terms of function. Of the four terminally ligating Cys residues, surprisingly only substitution at ßCys-153 is tolerated with respect to retaining any significant spectroscopic and catalytic activity. Even though αCys-154 occupies an almost equivalent position on the other $[Fe_4S_4]$ sub-cluster (with both residues on the surface side of the prosthetic group that is possibly involved in accepting electrons), all tested substitutions here result in the loss of all activities. Possibly, the location of ßCys-153 furthest away from FeMoco indicates that it has a less structurally and functionally demanding role in intramolecular delivery of electrons to FeMoco. In addition, ligation of the same Fe atom by ßSer-188 might be an important factor. The strict requirement for (and, therefore, intolerance to substitution by) αCys-62 might be explained by its closest approach to FeMoco (ca. 14Å) and its involvement in one of several helices, which are potential electron-transfer pathways, that span from P-cluster pair to FeMoco. The structures and models offer no explanation for the requirement for αCys-154 and ßCys-70. Both of the bridging ligands, αCys-88 and ßCys-95, are also part of prosthetic group-spanning helices and, therefore, of potential electron-transfer pathways. However, only substitutions at αCys-88 retain significant activities. Again, there is no obvious structural difference among them except that αCys-88 is further from FeMoco and, therefore, possibly plays a less demanding catalytic role.

The ßC153S- and ßS188G-MoFe proteins have been purified to homogeneity. The only differences from wild type in high-flux steady-state assays are their maximum specific activities for N_2, H^+ and C_2H_2 reduction, which are ca. 50% of wild type (May et al., 1991) and slightly elevated ATP/2e$^-$ levels. All spectroscopic tests of FeMoco show it to be unchanged. EPR and MCD (magnetic circular dichroism) of both thionine-oxidized altered proteins indicate that the properties of the P-cluster pair have been affected. Stopped-flow spectrometry indicates that a similar, possibly tighter, complex is formed with the Fe protein (dissociation rate of 5 s^{-1} vs. 6 s^{-1}; K_d(MgATP) smaller; similar efficiency in oxidizing Fe protein). Only the primary electron-transfer rate (100 s^{-1} vs. 200 s^{-1} for wild type) is significantly changed for these two MoFe proteins but, because this rate is still so much faster than the dissociation rate, which is rate-determining in wild type, this difference cannot explain the lowered maximum specific activity for substrate reduction. Some, as yet unknown, process internal to the MoFe protein has apparently become rate-limiting.

Several substitutions at αCys-88 result in altered MoFe proteins retaining the ability to fix N_2. Three, αC88D-, αC88G-, and αC88T-MoFe proteins, have been purified. Spectroscopic (EPR, MCD and Mössbauer) and catalytic probes indicate that the FeMoco centers of all three proteins are uncompromised, but the properties of their P-cluster pairs have changed. Of most interest is the observation that, in the dithionite-reduced samples of αC88G- and αC88T-MoFe proteins, the P-cluster pairs appear predominantly in their one-electron oxidized $[Fe_8S_8]^+$ redox state with EPR parameters consistent with those seen during dye oxidation of wild type (Tittsworth, Hales, 1993). This change in redox properties of these P-cluster pairs is consistent with the changed electron-transfer and protein-protein interaction parameters of the altered MoFe proteins that contain them. These studies indicate that the P-cluster pair is the likely recipient of electrons transferred from the Fe protein, but do not rule out some other additional role in catalysis.

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PHOTOSTIMULATION OF NITROGENASE REACTION IN VITRO FOR INVESTIGATION OF NITROGENASE MECHANISM ACTION

SYRTSOVA L.A., DRUZHININ S.Yu., KHRAMOV A.V.,
MORAVSKY A.P.
Institute of Chemical Physics of Russian Academy of Sciences,
Chernogolovka, Moscow Region, 142432

Nitrogenase is classified as an enzyme of energy coupling. The reaction of ATP hydrolysis in nitrogenase is coupled to reaction of intramolecular electron transfer against the potential from Fe protein (Av2) to MoFe protein (Av1) with the formation of so-called Av1 superreduced state, a process preceding the substrate reduction (Smith et al., 1988). In vitro for stimulation of nitrogenase turnover the artificial electron donor, sodium dithionite was commonly used. Unfortunately, with use of this donor the step of nitrogen reduction is not rate-limiting and can not be studied by conventional methods of enzymatic kinetics. Analysis of the kinetic data on nitrogenase turnover showed that dissociation of the enzyme into its components after the electron transfer from Av2 to Av1 ($k=6,4\ s^{-1}$ at 295 K) was the rate-limiting step for the system employing dithionite as a donor (Thorneley, Lowe, 1983). However, dithionite is characterized by high Michaelis constant value (9 mM; Burns, 1969) and was commonly used at relatively high concentrations: >10 mM. As shown by Eady (1973), the nitrogenase complex does not persist in the presence of such dithionite concentrations. Therefore it was not quite clear whether dissociation of nitrogenase into its components is a natural process or an artifact, caused by the high dithionite concentration. To develop the appropriate methods for the studies of the kinetics of nitrogen reduction by nitrogenase the alternative photochemical electron donor system for nitrogenase reduction, eosin + NADH in the light, had been proposed earlier (Druzhinin et al., 1993). This donor system renders possible the registration of the kinetics of nitrogenase turnover in ms-range. Photochemical reduction of nitrogenase was more effective than the activity in the presence of dithionite. More than 2-fold increase in the total electron flux through nitrogenase as compared with dithionite system was observed in the presence of eosin-NADH in the light at Av2:Av1=1. One hypothesis for the observed acceleration was that the dissociation does not take place in the presence of the photochemical system. Thus, to determine the limitations of nitrogenase catalysis, the stoichiometry of an active nitrogenase complex and the possibility of its dissociation during turnover in the presence of the photochemical donor system have been studied.

Preparation of crystalline Av1 and homogeneous Av2 from *Azotobacter vinelandii OP* and determination of specific activity of nitrogenase components were as described in a

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previous papers (Syrtsova et al., 1983; Druzhinin et al., 1993). For measurement of fast change of eosin fluorescence and that of nitrogenase absorption an earlier described stopped-flow apparatus was used (Moravsky, Khramov, 1984; Syrtsova et al., 1988). The electron transfer rate from Av2 to Av1 was determined through the measurement of the temporal change of absorbance at 420 nm after mixing the system with MgATP in the stopped-flow cell, following the method of Eady et al. (1978).

IDENTIFICATION OF Av2 IN SOLUTION IN THE PRESENCE OF Av1.

For implementing of two formulated above problems, the necessity of development of a method for following the concentration of free Av2 in solution in the course of nitrogenase turnover in the presence of Av1 has been raised. The basis for the approach used was the reaction, specific for Av2, of interaction with O_2 of about an equimolar concentration, in ms-range. Under mixing by stopped-flow technique, of the solution of 4-20 μM Av2, containing 0,2-0,8 mM dithionite with Tris-buffer, containing 5 μM O_2 , the fast Av2 oxidation occurs in the early period. But owing to exhaustion of O_2 in the reactions with Av2 and dithionite, the oxidation of Av2 is succeeded by its re-reduction by dithionite (Fig. 1, a). For enhancement of the method sensitivity, 5-10 μM eosin was introduced into the reaction mixture. In this case the redox changes, depicted in Fig. 1, a, can be followed by the eosin fluorescence. As it is known, Av2 has a broad absorption spectrum that overlaps with the spectrum of the eosin fluorescence. This situation is a necessary condition for the inductive - resonance energy transfer by Forster (1948) - Galanin (1951). So the change of Av2 absorption during the change of its redox state (Fig. 1, a) must influence the fluorescence of eosin, interacting with Av2. In line with this assumption, Figure 1 shows the simultaneous changes of Av2 absorption and of eosin fluorescence. In the moment of mixing, Av2 quenched the eosin fluorescence by about 20 % with $k>1000\ s^{-1}$. In the absence of O_2 the changes of eosin fluorescence and of Av2 absorption were not observed. The interaction of eosin with nitrogenase was described earlier (Druzhinin et al., 1993). 5-10 μM eosin does not inhibit the nitrogenase turnover. Because of much higher signal / noise ratio, the fluorescence records are preferable for kinetics analysis. Therefore in the paper given the fluorescence was studied as a rule. Both processes of the rapid change of eosin fluorescence (Fig. 1,b) can be approximated by the exponential. Moreover, the peak amplitude of increase of eosin fluorescence was linearly proportional to Av2 concentration, enabling to plot a calibration curve for determination of the Av2 concentration. The choice of the dithionite concentration was determined from the dependence of the rate constant of the rapid fluorescence increase, k_2 , (Fig. 1, b) on the dithionite concentration. So far as it was proposed to investigate the slow processes ($k \sim 6,4\ s^{-1}$), the dithionite concentrations 0.3-0.4 mM were used, securing the rate constants, k_2, in range about $100\ s^{-1}$. As it is known such dithionite concentrations do not cause dissociation of the nitrogenase complex (Eady, 1973). Upon an increase in the dithionite concentration up to $10^{-3}M$, neither rapid changes of fluorescence or redox conversions of Av2 are observed. In this case, O_2 is consumed more rapidly by reaction with dithionite, than with Av2. At longer times (0-1 s, 0-10 s or 0-100 s) the slow exponential decay of eosin fluorescence has been recorded.

The last process is caused by the interaction of eosin with 0.1-0.4 mM dithionite: k= (1,2-1,4)x10^{-2} s^{-1} at 293 K, as the control tests show. On study of Av2 inactivated by O_2 in air, only the last slow process was observed. It is noteworthy that the intensity of exciting light in the experiments is inadequate to support the process of nitrogenase photoreduction. Therefore the other component of photochemical system, NADH has no influence on the processes observed in Fig. 1, and can be absent in reaction mixture.

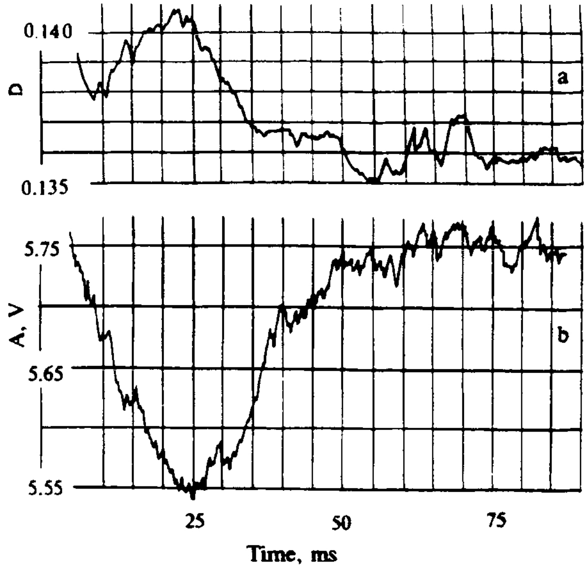

Figure 1. Changes of the optical density (a) and of fluorescence intensity (b) after rapid mixing of 20 µM Av2 with eosin and 5 µM O_2 at 293 K. The first syringe contained 20 µM Av2, 0.68 mM dithionite and 0.03 M NaCl. The second syringe contained 10 µM eosin and 5 µM O_2. The solvent is 0.025 M Tris-buffer, pH 7.2.

In the contrast to Av2, Av1 does not interact with O_2 in conditions of experiment, depicted in Fig. 1, a. Therefore in the case of rapid mixing of Av1, containing the dithionite, with a solution of eosin, containing 5 µM O_2, under the conditions of stopped-flow experiments, we observed only the slow exponential decrease in fluorescence with k = (1.3± 0.2)x10^{-2} s^{-1} at 293 K, corresponding to the interaction of the eosin and the dithionite. Thus, Av1 interacts with 2,5 µM O_2 more slowly, than the dithionite does in these conditions. In the contrast to Av2, in the moment of mixing, Av1 quenches completely the fluorescence of an equimolar quantity of eosin with the reaction rate constant exceeding 1000 s^{-1}.

PHOTOCHEMICALLY-DRIVEN NITROGENASE TURNOVER.

The present work applies the method for determination of free Av2 in solution for the investigation of nitrogenase components stoichiometry and the possibility of nitrogenase dissociation under the turnover reaction process. Nitrogenase at different ratios of Av2:Av1 in the presence of dithionite was mixed with the solution of eosin and MgATP containing 5 μM O_2 in a stopped-flow apparatus. The range of measurement of the kinetics of the eosin fluorescence varied within 0-100 ms, 0-1 s, 0-10 s, 0-100 s. In the experiments with Av2:Av1 ratio exceeding 1, the fluorescence changes of eosin similar to those in Figure 1 were observed (Table). The Av2 concentration in solution was determined with the aid of a calibration curve, correlating the concentration of free Av2, unbound to nitrogenase complex, to the amplitude of eosin fluorescence recovery A ; both sets of values were taken from entries 1-4 of Table. To compare the conditions of entries 5-9 (Table) to those of entries 1-4 (Table), the ability of Av1 to quench the fluorescence of one equivalent of eosin was taken into account. In this respect, double eosin concentration was used in experiments with Av2+Av1 complex (entries 5-9; Table), as compared to the experiments with Av2 as the sole protein reagent (entries 1-4; Table). That guarantees the same initial level of eosin fluorescence in the experiments with complex (Av1+Av2) and Av2. In all experiments with the mixtures of the nitrogenase proteins, the determined concentration of free Av2 was always equal to the difference in initial concentrations of proteins, [Av2]-[Av1]. As seen from Table, the mixture of Av1 and Av2 components (1:1) did not contain a detectable amount of free Av2. Therefore only Av2, which was not bound to Av1 in equimolar nitrogenase complex (i.e. free Av2) could be observed with certainty. In the presence of 0.2-20 mM NADH the same results were obtained. Hence in the presence of donor photochemical system under dithionite concentrations not exceeding 0.4 mM, the nitrogenase complex (Av2+Av1) of components ratio equal to 1:1 exists. Since the solutions of Av2 and Av1 proteins contained the dithionite in the quantities sufficient for their reduction, the intramolecular electron transfer from Av2 to Av1 occurred upon mixing nitrogenase with MgATP. Therefore in the experiments with Av2 and Av1 under ratio about 1 (entries 8,9, Table), in the absence of free Av2 in solution, only the enzymatic oxidation of Av2 occurred, followed by the fluorescence decrease with the rate constant k_1. In the control experiments, in the absence of eosin in reaction mixture, the electron transfer occurred with the same rate constant. There was no the electron transfer without MgATP, and in the experiments similar to 8,9 (Table) the fluorescence changes were not observed. In the experiments with nitrogenase complex (Av1+Av2) (entries 5-9, Table) k_1 is the total rate constant of the process of Av2 oxidation: both by O_2 and /or intramolecular electron transfer from Av2 to Av1. It is noteworthy to emphasize that the dithionite did not reduce Av2, bound in the complex with Av1, as it is known from Thorneley, Lowe (1983). Therefore in experiments 8,9 (Table) the process of the fluorescence increase was absent. The latter situation is responsible for determination of free Av2 concentration in the presence of complex (Av1+Av2). The experiments with nitrogenase complex (entries 5-9, Table) permit to investigate the possibility of nitrogenase dissociation into components after electron transfer from Av2 to Av1. It was established that the rate constants of registered process of fast increase of eosin fluorescence (k_2, Table) in experiments with nitrogenase complex (entries 5-9, Table) are close to the constants of the corresponding process in the experiments with Av2 (entries 1-4, Table). Note that the above presented description of the character of the fast processes is to some extent simplified. In particular, the phenomenon of the protective (screening) action of Av1 towards Av2 (Bulen, LeComte, 1972) was ignored. This process may cause the observed k_1 and k_2 values to deviate from constancy by a factor of about 2 on increasing the Av2 concentration (entries 5-8, Table). In all experiments the slow, $k = (1\text{-}3)\text{x}10^{-2}\ s^{-1}$ decrease of eosin fluorescence was observed, caused by interaction of eosin with dithionite. The slow process of fluorescence increase with rate constant close to $k = 6{,}4\ s^{-1}$ was not registered. The lack of fluorescence increase in the range of 5 ms - 10 s strongly suggests the absence of the process of nitrogenase complex (Av1+Av2) dissociation into separate components Av1 and Av2 after electron transfer from Av2 to Av1 in the course of nitrogenase turnover, since even for a slow dissociation the recovery of fluorescence would be safely detected because of the known (Figure 1.a) fast reduction

TABLE. Determination of the nitrogenase complex stoichiometry and the possibility of nitrogenase dissociation during catalytic turnover by using stopped-flow spectrophotometry at 293 K. The first syringe also contained 0.74 mM dithionite; the second syringe also contained 2 mM MgATP and 5 μM O_2. k_1 and k_2 are the rate constants of the fast decrease and subsequent increase of fluorescence intensity, respectively. All solutions contained 0.025 M Tris - buffer and 50 mM NaCl.

N°	Content of reagents before mixing			Parameters after mixing				
	1st syringe		2nd syringe	k_1, s^{-1} ±10%	k_2, s^{-1} ±10%	A*	Free [Av2], μM	[Av2]**/ [Av1]
	[Av1] μM	[Av2] μM	[Eosin], μM					
1.	-	6	10	140	250	42±2	3±0.3***	-
2.	-	10	10	160	190	66±3	5±0.5***	-
3.	-	13.8	10	110	190	120±6	6.9±0.7***	-
4.	-	18	10	90	140	152±7	9±0.9***	-
5.	10	20	20	43	110	87±4	5.6±0.5	0.88:1
6.	10	16	20	59	100	41±2	2.6±2	1.08:1
7.	10	13.8	20	64	100	17±1	1	1.18:1
8.	10	12	20	46	0	0	0	1.2:1
9.	10	10	20	40	0	0	0	1:1
							Average	(1.07+0.1) :1

A^* = amplitude of the fluorescence increase, a.u.**[Av2]/[Av1] = protein ratio in nitrogenase complex (estimated).***= one half of Av2 concentration in the first syringe.

of liberated oxidized Av2 by dithionite. The experiments were repeated at least 6 times with 0.3-0.4 mM dithionite in reaction mixture with the same results.

CONCLUSIONS.

Thus, the data presented here provide direct experimental proof for the persistence of nitrogenase complex (Av1+Av2) of ratio equal to 1 in the presence of donor photochemical system. This complex does not dissociate after the electron transfer from Av2 to Av1 with constant $0.1\ s^{-1} < k < 200\ s^{-1}$. In such a complex Av2 can not randomly interact with two independent center in Av1, as it takes place with donor dithionite (Hageman, Burris, 1978-), and the rate-limiting step can be the real one of the nitrogen reduction. The analogy can be noted with the behavior of nitrogenase in vivo. The specific activity of nitrogenase in vivo is higher about 2-fold in comparison to activity in vitro with dithionite. With the natural donor, flavodoxin in vitro the activity is also higher (Klugkist et al., 1985). Therefore the use of donor photochemical system in the presence of complex (Av1+Av2) 1:1 guarantees the conditions for the study of nitrogen reduction, corresponding to conditions in vivo.

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ATP HYDROLYSIS AND ENERGY TRANSDUCTION BY NITROGENASE

*LOWE D.J., *ASHBY G.A., †BRUNE M., *KNIGHTS H., †WEBB M.R., *THORNELEY R.N.F.
*Nitrogen Fixation Laboratory, University of Sussex, Brighton BN1 9RQ, UK.
†National Institute for Medical Research, Mill Hill, London NW7 1AA, UK.

1. INTRODUCTION:

We have, in the past, described the functioning of molybdenum nitrogenase in terms of a kinetic model that describes the interactions between the two component proteins (the Fe-protein and the MoFe-protein), reductant dithionite, substrates and products (Lowe, Thorneley, 1984a,b; Thorneley, Lowe, 1983, 1984a,b). In this cycle ATP hydrolysis is associated with electron transfer from Kp2 to Kp1 followed by obligate, rate-limiting, dissociation of the protein complex to give reduced Kp1 and free oxidised $Kp2_{ox}(MgADP)_2$. Phosphate release was arbitrarily assigned to the protein complex dissociation step. Eight Fe-protein cycles, each involving the transfer of an electron to the MoFe-protein, combine to give a single MoFe-protein cycle in which a molecule of dinitrogen and eight protons are reduced to two molecules of ammonia and one of dihydrogen. The rates of all the partial reactions involved in these reactions have been independently measured.

In this paper we wish to address the hydrolysis of ATP by nitrogenase in terms of our Fe-protein cycle (Figure 1, cycle (a)). Under steady-state conditions the enzyme hydrolyses at least two molecules of ATP in a complex with a divalent metal ion, usually Mg^{2+}, per electron transferred to reducible substrate. In addition to the Fe-protein cycle of nitrogenase Figure 1 also shows, in cycle (b), reactions that occur in the absence of reductant which also hydrolyse ATP rationalising the observations that, under a number of non-optimal conditions, the efficiency of coupling of ATP hydrolysis to electron transfer can be much higher (Jeng et al, 1970; Imam and Eady, 1980; Watt et al, 1975; Hageman and Burris, 1978). Note that in both cycles (a) and (b) ATPase activity is a property of a complex involving both proteins and not of either protein when isolated. The two putative ATP binding sites identified on the *Azotobacter vinelandii* Fe-protein by X-ray crystallography (Georgiadis et al, 1990) have been generally assumed to be the sites of MgATP hydrolysis. The simulations of the dependence of the rate of dihydrogen evolution on dithionite concentration support this assignment since they depend critically

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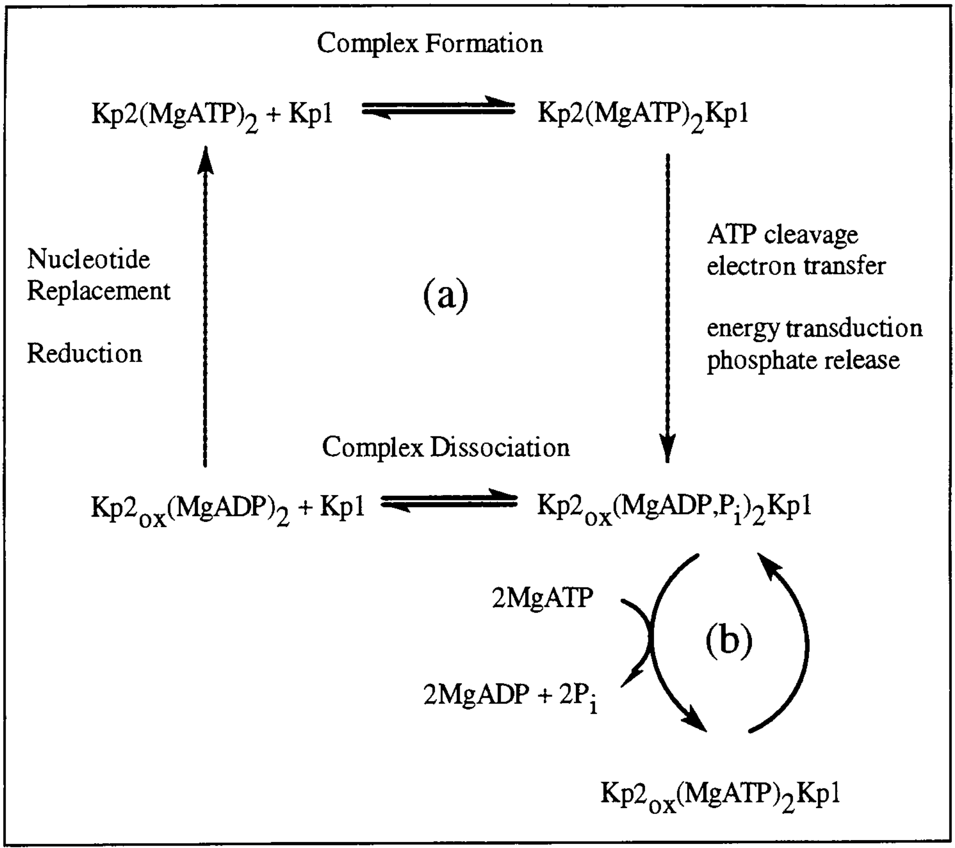
Complex Formation
$Kp2(MgATP)_2 + Kp1 \rightleftharpoons Kp2(MgATP)_2Kp1$
Nucleotide Replacement
Reduction
(a)
ATP cleavage
electron transfer
energy transduction
phosphate release
Complex Dissociation
$Kp2_{ox}(MgADP)_2 + Kp1 \rightleftharpoons Kp2_{ox}(MgADP,P_i)_2Kp1$
2MgATP
2MgADP + $2P_i$
(b)
$Kp2_{ox}(MgATP)_2Kp1$

Figure 1. The Fe-Protein cycle of Nitrogenase. Kp1 and Kp2 are the MoFe- and Fe-proteins; cycles (a) and (b) show reductant dependent and independent ATP hydrolysis.

on the rate of reduction of $Kp2_{ox}$ in each Fe-protein cycle and show that $Kp2_{ox}(MgADP)_2$ must be the species reduced by the $SO_2^{\cdot-}$ derived from dithionite ion (Thorneley and Lowe, 1984b). There is increasing evidence for MgATP/ADP binding sites on the MoFe-protein although their significance is not yet understood (Miller et al, 1993).

The overall process of ATP hydrolysis by nitrogenase has been studied using a number of techniques including pre-steady-state rapid quench of turning over enzyme to monitor formation of phosphate (Eady et al, 1978; Hageman et al, 1980), pre-steady-state proton production using pH indicators (Mensink et al, 1992), mass spectrometry (McKenna et al, 1989), stopped-flow calorimetry (Thorneley et al, 1989) and directed mutagenesis (Seefeld et al, 1992). There are problems with unambiguous interpretation of these data: rapid acid quench cannot distinguish between on-enzyme cleavage of ATP and subsequent phosphate release whereas heat and pH changes are difficult to assign to a particular partial reaction. Our original formulation of the Fe-protein cycle rationalised interactions between the two proteins, i.e. complex formation and dissociation as well as electron transfer. Under our experimental conditions ATP was always saturating and its hydrolysis was not considered, although the pre-steady-state studies mentioned above had indicated that it was closely associated with intermolecular electron transfer (the step in Figure 1 from $Kp2(MgATP)_2Kp1$ to $Kp2_{ox}(MgADP,P_i)_2Kp1$). It is important to remember that ATP hydrolysis is a process that requires a number of steps including: binding of MgATP; on-enzyme cleavage of MgATP to MgADP and phosphate; release of inorganic phosphate; release of MgADP. Any of these steps could, in principle, involve proton transfer, proton release, electron transfer and energy transduction. In the study described here, we use a newly developed direct, real-time assay for rapidly measuring inorganic phosphate release using a fluorescent probe (Brune et al, 1994). This technique enables us to monitor phosphate release from nitrogenase with a sensitivity of about 10 nM on the millisecond time scale. We are thus able to analyse MgATP hydrolysis with a greatly increased resolution. Note that phosphate release is frequently found to be the 'work' step, where energy transduction occurs, for a number of ATPases (Thorneley, 1992).

2. PRE-STEADY-STATE PHOSPHATE RELEASE BY NITROGENASE

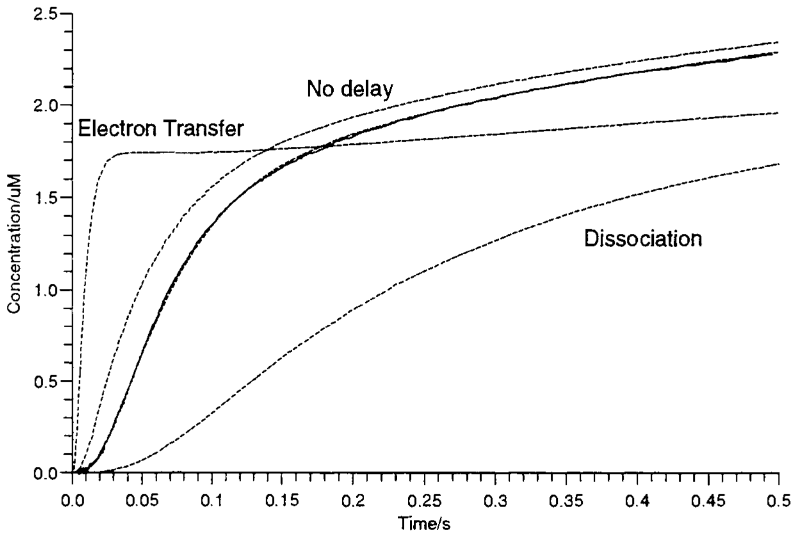

Figure 2. Pre-Steady-State Phosphate Release from Nitrogenase. The solid line shows the data and the dotted lines show simulations with various assumptions given in the text.

The solid line in Figure 2 shows the first 500 ms of the time course for release of inorganic phosphate produced by MgATP hydrolysis during nitrogenase turnover monitored by the increase in fluorescence of the phosphate probe in a stopped-flow apparatus. The experiment was done at 23°C in an anaerobic glove box using 2μM Kp1 and 6μM Kp2 in 25 mM HEPES buffer pH 7.4 containing sodium dithionite (0.52 mM), $MgCl_2$ (10 mM) and ATP (2 mM); all concentrations are given after mixing of the solutions. The data clearly show a lag of about 20 ms, then a burst lasting about 100 ms followed by an approach to a steady-state rate.

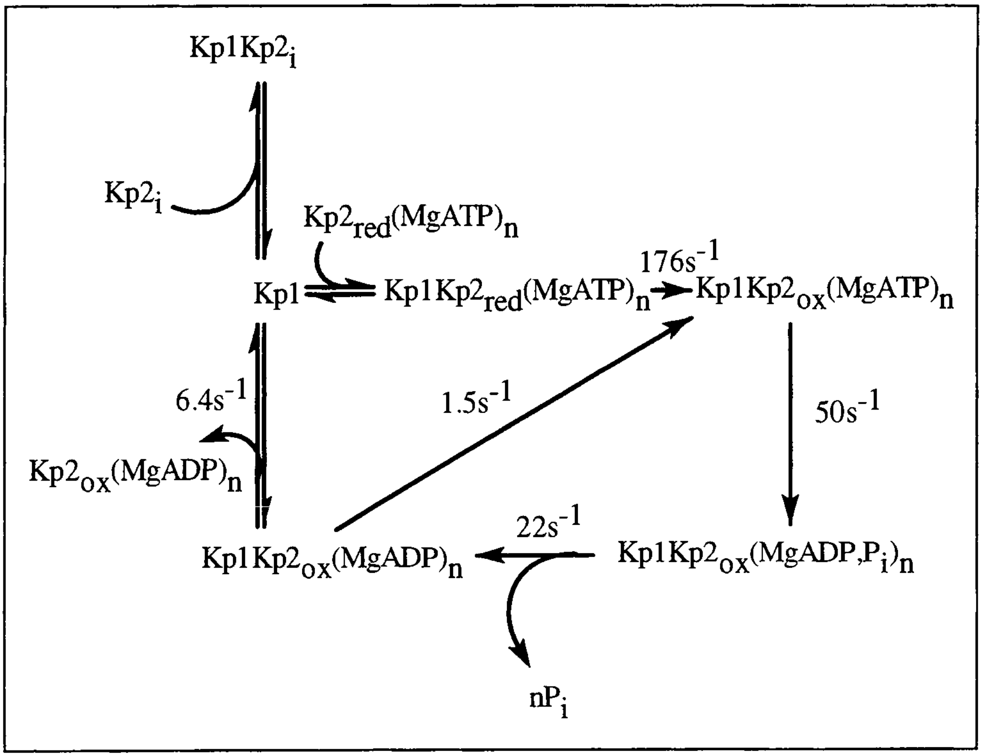

Figure 3. Kinetic Scheme used for Simulating Pre-Steady-State Phosphate Release from Nitrogenase.

The dotted lines in Figure 2 are simulations made using the scheme of Figure 3 under different assumptions about where in the Fe-protein cycle the phosphate is released. Figure 3 is an expansion of our Fe-protein cycle. Clearly the simulations assuming that phosphate is released concomitant either with electron transfer between the proteins (at 176 s^{-1}) or with dissociation of the protein complex (at 6.4 s^{-1}) are inconsistent with the data. We have thus identified two new partial reactions, one of which is a delay and is followed by phosphate release. The delay step is necessary to fit the data since omitting this produces fits of the form given by the 'No delay' curve of Figure 2. Under our conditions both these processes must occur after intermolecular electron transfer but before protein-protein dissociation.

It is interesting to note the stoichiometry of phosphate release. If we assume that only the active Fe-protein (which constitutes 45% of the total) is involved in MgATP hydrolysis, then the number of phosphates released per cycle (n in Figure 3) is 2.2. However if we also include inactive Fe-protein ($Kp2_i$) n falls to 1.0.

3. DISCUSSION:

Why does nitrogenase hydrolyse ATP? Our new results provide some insights into this question. Speculation about its role has included: 1) the gating and coupling of proton and electron transfers (note that all the reductions catalysed by nitrogenase involve addition of both); 2) the generation of lowered redox potentials at one or both of the two kinds of metal clusters in the MoFe-protein (the so-called ‘P’-clusters and the presumed substrate reduction site, FeMoco); 3) the induction of the dissociation of the Fe-protein:MoFe-protein complex.

Firstly, since phosphate bound to $Kp2_{ox}(MgADP)_2Kp1$ complex is not required to obtain the protein:protein dissociation rate of 6.4 s^{-1} (Thorneley, Lowe, 1983), it is clear that energy transduction does not drive the dissociation process. Secondly we need to remember that under the conditions used in the experiments described here (at 23°C) we still do not know the rate of on-enzyme cleavage of ATP to ADP,P_i. If the assignment, by analogy with myosin, of the endothermic process at 5°C to the on-enzyme cleavage reaction is correct (Thorneley et al, 1989) electron transfer at this temperature apparently occurs more slowly than cleavage, but without the lag phase that would be required for a sequential process. In order to rationalize our 23°C and 5°C data with that of Mensink et al. (1992) it seems likely that either the MgATP or MgADP,P_i conformation of the Fe-protein is all that is required for electron transfer to happen and that concomitant with phosphate release a key energy transduction event occurs. One can speculate on what this might be. Perhaps it is the modulation of the redox potential of ‘P’-clusters and/or of FeMoco. Perhaps it is related to gated proton transfer to the substrate reduction site. What is clear however is that, if MgATP hydrolysis is occurring at the site on the Fe-protein identified by Georgiadis et al (1990), the Fe-protein:MoFe-protein interface must play a crucial part in transducing the free energy of phosphate release from the Fe-protein to the metal clusters in the MoFe-protein. The concerted nature of the electron transfer, MgATP hydrolysis and protein:protein dissociation reactions has been revealed by the proportional decrease (*ca.* 6-fold) in the rates of all these reactions on replacing the interfacial residue β-Phe 124 of the MoFe-protein by Ile (Lowe et al, 1993). A similar effect is also observed on replacing ATP by 2’-deoxyATP (Knights, Lowe, Thorneley, unpublished). These effects are strongly indicative of a simultaneous, concerted conformational change of both proteins within the nitrogenase complex, presumably triggered by phosphate release.

4. ACKNOWLEDGEMENTS:

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CO INHIBITION OF NITROGEN FIXATION

CAMERON, L. M. AND HALES, B. J.
Department of Chemistry, Louisiana State University, Baton Rouge, LA 70803-1804 USA

In the absence of added substrate, such as C_2H_2 or N_2, Mo-nitrogenase reduces H^+ to H_2 (Burgess, 1985). CO strongly inhibits the reduction of all external substrates but does not affect H_2 evolution (Burgess, 1985). We've investigated the effect of CO on acetylene reduction activity from of V-nitrogenase *Azotobacter vinelandii* and found that CO similarly inhibits all substrate reductions except for H^+ to H_2. However, CO is not as potent an inhibitor of V-nitrogenase, exhibiting a K_i (3 kPa) approximately 100 times greater than that of Mo-nitrogenase. A similar K_i had been determined previously for V-nitrogenase from *Azotobacter choococcum*. Since the only known difference between the metal clusters of the Mo and V forms of the enzyme is the presence of these heterometals, this result suggests that CO may be binding to V or Mo in the cofactor. However, our recent results (Moore, et al., 1994) with hybrid nitrogenase discussed below call the suggestion of CO binding to the cofactor into question.

We recently have observed several new and interesting properties of CO inhibition in both Mo and V enzyme systems from *A.vinelandii* . One property concerns the induction of EPR signals by CO. During enzymatic turnover in the presence of CO, Mo-nitrogenase generates 2 strong transient $S = 1/2$ EPR signals (Davis, et al., 1979; Lowe, et al., 1978). One of the CO-induced signals (lo-CO) is produced under low CO pressures ($P_{CO} < 1$ kPa) and is replaced by a second signal (hi-CO) as the CO pressure increases. Enrichment of Mo-nitrogenase component 1 with ^{57}Fe produces a broadening of these signals (Davis et al., 1979), suggesting that they originate in either the M-center or P-cluster of that protein. We've conducted similar turnover experiments using V-nitrogenase instead of Mo-nitrogenase and a gas phase with $P_{CO} = 101$ kPa, but detected no CO-induced EPR signals. Furthermore, hybrid component 1 [Av1'(FeMoco)] containing the polypeptides of V-nitrogenase (Av1') with the cofactor of Mo-nitrogenase (FeMoco) does not express either CO-induced EPR signal and has a K_i for acetylene reduction identical to Av1'.

The requirement of enzymatic turnover for the observation of the CO-induced signals implies that that these signals do not occur in the resting state ($\mathbf{E_0}$ in the Lowe-Thorneley model (Lowe & Thorneley, 1984a; Lowe & Thorneley, 1984b; Thorneley & Lowe, 1984a; Thorneley & Lowe, 1984b)) of Mo-nitrogenase, but in a turnover state $\mathbf{E_n}$ with $\mathbf{n} \geq 1$. According to the Lowe-Thorneley (L-T) model, states $\mathbf{E_2}$ and $\mathbf{E_3}$ have the ability to relax to $\mathbf{E_0}$ and $\mathbf{E_1}$, respectively, with the evolution of H_2. This relaxation is a minor process during normal catalytic activity under high electron flux conditions, but becomes the dominant rate process under conditions of low electron flux. Electron flux is the rate of

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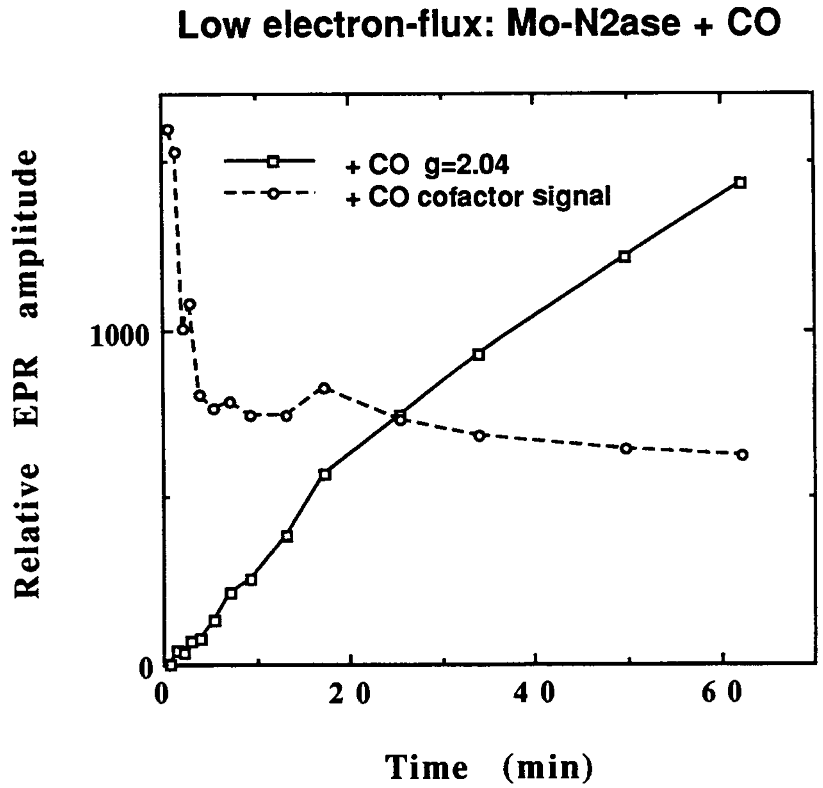
electron transfer from component 2 through component 1. Low electron flux can be achieved by having a low ratio of Av2 to Av1. Because of the relatively small amount of component 2, there is a long time delay between each electron transfer step to component 1. The L-T model predicts that once the enzyme reaches state E_2, it is either reduced further to E_3 or relaxed back to E_0 with evolution of a molecule of H_2. During low electron flux the relaxation rate dominates and E_0 and E_1 are the only states with significant populations (Fischer, et al., 1991).

To determine which state(s) generates the CO-induced EPR signals we prepared a turnover mixture of Mo-nitrogenase in a 1:50 molar ratio of Av2 to Av1 (i.e., very low electron flux) and allowed it to incubate for 60 min. At various time intervals during the incubation small samples were extracted, placed into anaerobic EPR tubes and rapidly frozen in cold isopentane. The figure below shows the amplitude profile of both the cofactor $S = 3/2$ signal (E_0 state) as well as the hi-CO ($g = 2.04$) signal as a function of time.



In the absence of CO and during low electron flux and because the state E_1 is EPR silent, the cofactor signal (E_0 state) normally deminishes to about half its original amplitude during turnover (Fischer et al., 1991). As seen in the above graph, in the presence of CO the cofactor signal also reaches a steady state at approximately half its original amplitude. However, during this same time period, hi-CO grows in slowly but continuously even after the cofactor signal has reached a steady-state concentration. This graph shows several important features of the CO-induced EPR signal. First, there is a slight lag in the induction of the signal suggesting that the CO-induced signal is slowly generated in state $\mathbf{E_2}$ or possibly $\mathbf{E_3}$. Second, the slow but continuous increase of the CO-induced signal with time implies that once generated, the signal persists even after the enzyme has relaxed back to $\mathbf{E_0}$ or $\mathbf{E_1}$. It is important to remember that in these low-electron flux experiments, the spin concentration of the CO-induced signal is much lower than that of the cofactor signal. Therefore, the hi-CO signal in the above graph represents only a very small fraction of total protein. Because of this, the corresponding slow decrease in the cofactor signal is not as obvious from this plot. We have determined the spin concentration of both the cofactor and the CO-induced signal at different times in this experiment and find conservation of spin concentration. As the concentration of the CO-induced signal increases, the concentration of the cofactor signal decreases at the same rate, suggesting that the formation of the CO-induced signal is kinetically linked to the decrease in the cofactor signal. A similar dependency had been shown previously (Davis et al., 1979).

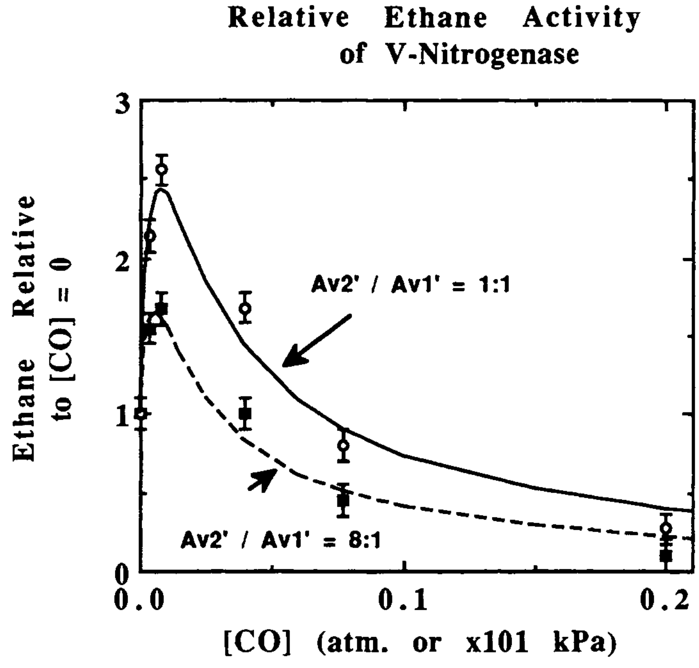
Upon termination of catalysis, the CO-induced signal decays while the cofactor signal returns. This result is consistent with CO binding only to the enzyme in a reduced state generated by turnover. In the past, this experiment was performed (Davis et al., 1979) by allowing the enzyme to undergo turnover in the presence of CO and limiting ATP. As ATP was consumed by the reaction, the rate of reaction would decrease, eventually terminating upon depletion of all the ATP. We were interested in determining what happens to the CO-induced EPR signals when the enzyme reaction was suddenly (rather than gradually) terminated. We've discovered that the addition of ethylene glycol (final concentration ≥40%) to the nitrogenase reaction mixture rapidly and completely quenchs the reaction. When ethylene glycol was added to an Mo-nitrogenase mixture in the presence 0.5 atm CO, the hi-CO signal persisted even though turnover was no longer taking place. Subsequent pumping on the sample to remove CO from the environment caused the hi-CO signal to convert to lo-CO. Suprisingly, introduction of CO back into the gas phase *regenerated* the hi-CO signal even though *enzymatic turnover was no longer occurring*. This experiment is very important and suggests that sudden quenching of the enzyme with ethylene glycol has "frozen" the protein into a conformation, different from that of the resting enzyme, which is able to reversibly interacts with CO, allowing it to generate the lo-CO and hi-CO signals.

A second interesting property of CO inhibition of nitrogenase that we've observed is the dependency of this inhibition on the rate of electron flux through the enzyme. The table below shows that the K_i (CO) for CO inhibition of C_2H_2 to C_2H_4 by either Mo- or V-nitrogenase depends on the ratio of component 2-to-component 1. In general, as the rate of electron flux increases, the effectiveness of CO as an inhibitor also increases. These results suggest that the enzyme exists in different conformations (possibly correcponding to different $\mathbf{E_n}$ states in the L-T model) where each conformation has a different affinity for CO. As the rate of electron flux through the enzyme increases, the enzyme's steady-state concentration shifts from one conformation to another (or from one $\mathbf{E_n}$ to another) resulting in the apparent change in K_i.

K_i (in kPa) for C_2H_2 to C_2H_4

Enzyme \ Component 2-to-Component 1 Ratio	1:1	8:1
Mo-Nitrogenase	0.40	0.02
V-Nitrogenase	30.0	3.0

Even more suprising is the effect of CO on the production of C_2H_6 from C_2H_2 by *A. vinelandii* V-nitrognease. The figure below shows that small concentrations of CO actually induce an enhancement of C_2H_6 production. As described above for the inhibition of C_2H_4 production, the magnitude of the enhancement of C_2H_6 also depends on the rate of electron flux, i.e., the degree of enhancement increases as the rate of electron flux decreases. This is the first example of CO not acting as an inhibitor of the reduction of an external substrate by a nitrogenase enzyme. Higher CO concentrations, however, do eventually inhibit reduction, suggesting that there are at least two different CO binding sites in V-nitrogenase.



From this result, a model can be formulated for CO binding to Av1' (**E**). In this model, CO (**I**) is able to bind to two different sites (**EI**$_1$ and **EI**$_2$) on the protein. Defining the following binding constants

$$\boldsymbol{K_{i1}} = [\mathrm{E}]\,[\mathrm{I}] / [\mathrm{EI}_1] \qquad \boldsymbol{K_{i2}} = [\mathrm{E}]\,[\mathrm{I}] / [\mathrm{EI}_2]$$

we can derive an expression for the amount of C_2H_6 produced (R) in the presence of CO relative to the amount when [CO] = 0.

$$\boldsymbol{R} = (1 + x\,[\mathrm{I}] / \boldsymbol{K_{i1}}) / (1 + [\mathrm{I}] / \boldsymbol{K_{i1}})(1 + [\mathrm{I}] / \boldsymbol{K_{i2}})$$

This model assumes that binding to site 1 (**EI**$_1$) at low CO concentrations enhances the production of C_2H_6 while binding to both sites (i.e., **EI**$_1$**I**$_2$) at higher CO concentrations inhibits its production. Allowing site 2 (**EI**$_2$) to also produced enhancement has no effect on the final simulation by this model. In this model, the degree of enhancement is represented by x. For example, if x = 2, the amount of C_2H_6 generated from acetylene reduction by **EI**$_1$ is twice that generated by the enzyme (**E**) in the absence of CO. Fitting programs show that the best simulations occur when $\boldsymbol{K_{i1}} = \boldsymbol{K_{i2}} = \boldsymbol{K_i}$ or

Component 2 / Component 1 Ratio	χ	K_i (in kPa)
8 : 1	5.4	0.9
1 : 1	8.6	1.0

Therefore, unlike the inhibition of acetylene to ethylene where the K_i dependent on electron flux, during the production of ethane the enhancement factor (x) depends on flux while the apparent K_i is flux independent. Furthermore, even though V-nitrogenase does not induce two different CO dependent EPR signals, as does Mo-nitrogenase, the enzymology of C_2H_6 generation by the V enzyme similarly suggests two different CO binding sites.

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NITROGENASE AND ITS CHEMICAL MODELS

SHILOV, A.E.
Institute of Chemical Physics of Russian Academy of Sciences, Chernogolovka, Moscow Region 142432, Russia

The structure of the FeMo-cofactor of nitrogenase allows us to draw conclusions about the importance of multiple contacts between dinitrogen and this polynuclear complex. Apparently, this arrangement corresponds to the maximum activation of dinitrogen and leads to the possibility of using comparatively weak reducing agents. Although there is no evidence for direct interaction of N_2 with iron atoms in cofactor, this is very likely, while vanadium and molybdenum in V- and Mo-dependent nitrogenases, respectively, stabilize the cluster and probably also regulate its redox potential. In model systems involving comparatively weak reducing agents, up to the present time, polynuclear complexes of Mo or V are the most active towards N_2. Iron-sulfur clusters are less stable and, so far, catalytic activity towards dinitrogen has been observed only in some cases with highly negative redox potentials.

With respect to acetylene, catalytic activity is observed both with polynuclear molybdenum and polynuclear iron complexes at the potential of Zn amalgam (E_0= -0,86 V) which is close to that of super-reduced FeMoco. In both cases, strong cocatalytic action of thiophenol is observed. The latter was chosen because of its relationship to the FeMo-cofactor of nitrogenase, which is held in the protein through the thiolate group of cysteine and whose EPR spectrum, after isolation from the protein, is modified by the presence of thiophenol to become similar to that of native FeMoco within the protein. For both model systems mentioned, the yield of the reduction products, ethylene and ethane, undergoes a sharp rise with thiophenol. It is particulary strong for ethane, while some methane, the product of six-electron reduction, was also observed.

The conclusion may be that, both in nitrogenase and in the model systems, the thiolate ligand mediates electron-transfer from either the double P-cluster in nitrogenase or from the zinc amalgam surface in the model systems, both of which donate electrons to the catalyst active center. Thus, the polynuclear character of the center helps to catalytically reduce N_2 and acetylene,while thiophenol assists the catalytic reduction, possibly by facilitating electron transfer to the active center.

So far, molybdenum and vanadium complexes are more active than iron-sulfur complexes. However, it might be anticipated that with a more appropriate conformation of the metal atoms in the cluster and a corresponding choice of ligands much more active systems, based on iron complexes, will be found.

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MECHANISTIC STUDIES ON IRON-SULFUR-BASED CLUSTERS

HENDERSON, R.A.
John Innes Centre, Nitrogen Fixation Laboratory, University of Sussex, Brighton
BN1 9RQ, UK

In order to understand how the nitrogenases work at the atomic level, it is essential that the fundamental chemistry underlying the reactions of the enzymes is well defined (Evans et al, 1993). The chemistry that has to be addressed falls into two headings: the transformations of the substrates and the reactivity of iron-sulfur-based clusters (Figure 1).

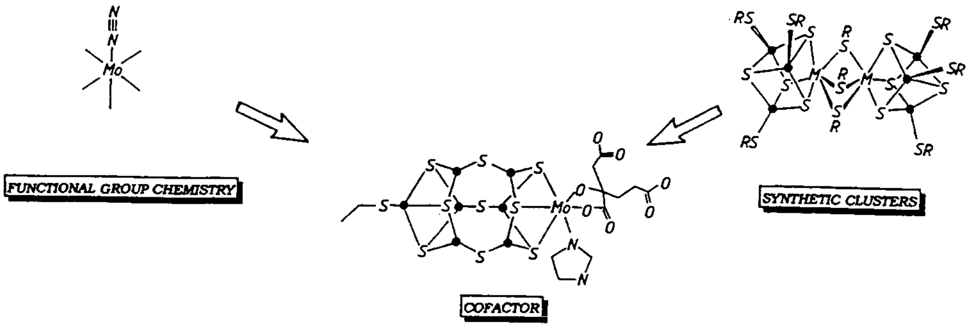

FIGURE 1

Work on simple mononuclear and binuclear complexes containing the nitrogenase substrates has defined in detail the electron- and proton-transfer pathways by which molecules such as dinitrogen, alkynes and isocyanides are converted into products.

However, all the metals in the nitrogenases are associated with iron-sulfur-based clusters. Many clusters of this general type have been prepared in the laboratory (Holm 1981) but the reactivity of this class of compounds is only poorly understood. Even where transformations of small molecules at clusters have been observed the pathway by which they are accomplished is far from clear. If the reactivity of the cofactor is to be understood then the general reactivity of iron-sulfur-based clusters must be defined. In particular we need to appreciate: (i) why biology uses a cluster to bind and activate substrates; (ii) where does dinitrogen or the other substrates bind on this cluster and; (iii) in what way does one metal affect the reactivity of other metals within the cluster? In the remainder of this article some of the general mechanistic characteristics for structurally well-defined iron-sulfur-based clusters will be highlighted. Characteristics that are so general we can be reasonably sure that they will be operating in the cofactor.

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THE SYSTEM. In order to study the intimate substitution mechanisms of this class of compounds the general system (Figure 2) has been developed (Henderson and Oglieve 1993a).The advantages of this system are: (i) all types of iron-sulfur-based clusters can be studied under comparable conditions; (ii) the relative proportions of RS^- and $NHEt_3^+$ can be controlled; (iii) substitution of only the first cluster ligand is monitored and; (iv) in MeCN as the solvent, the nature of the solution species can be quantified (*eg* acid strengths etc).

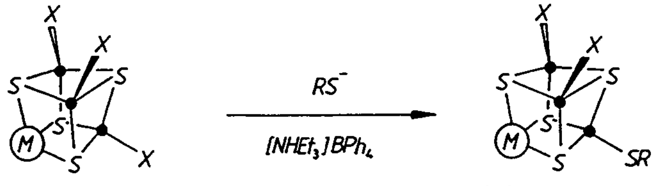

FIGURE 2: system used to study substitution mechanisms of clusters; M = Mo or Fe, ● = Fe.

SUBSTITUTION MECHANISMS. Studies on the substitution reactions of a variety of clusters including: $[Fe_2S_2Cl_4]^{2-}$, $[Fe_4S_4Cl_4]^{2-}$, $[Fe_4S_4(SR)_4]^{2-}$ (R = alkyl or aryl), $[MoFe_3S_4Cl_3(C_6Cl_4O_2)(NCMe)]^{2-}$, $[\{MFe_3S_4X_3\}_2(\mu\text{-}SR)_3]^{3-}$ (M = Mo or W; X = Cl or SR) and $[Fe_6S_6(OC_6H_4Me\text{-}4)_6]^{3-}$ have established the intimate substitution mechanism at iron centres in these clusters. In general, both associative and dissociative pathways can operate (Henderson and Oglieve 1993a,b, 1994a; Grönberg and Henderson unpublished) (Figure 3).

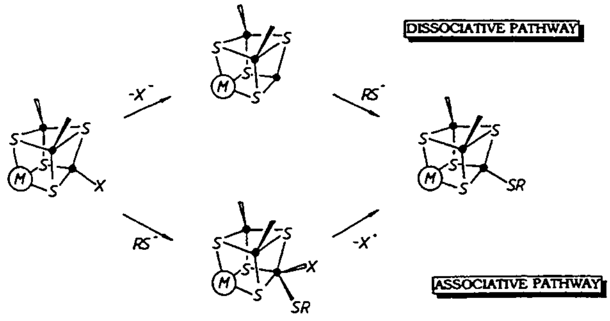

FIGURE 3

The intimate mechanism of the associative pathway involves rapid binding of the nucleophile to the cluster prior to the rate-limiting dissociation of the leaving group. At high concentrations of the nucleophile the intermediate on the associative pathway accumulates in stoichiometric amounts. The intermediate on the dissociative pathway is not detectable.

These substitution reactions are associated with large spectroscopic changes. In contrast, equally important cluster-based reactions (such as the binding of small molecules or protonation of the intact clusters) are not associated with such good spectroscopic handles. However, by coupling the protonation reactions, or the interaction of small molecules, with the substitution reactions we can get information about these other processes.

SITES OF PROTONATION. It was established some time ago (Dukes and Holm 1975) that acids catalyse the rate of substitution of iron-sulfur clusters. Using our system we have been able to show that: (i) protonation of these clusters is rapid, and the subsequent dissociation of the leaving group is the rate-limiting step in the acid-catalysed reactions and; (ii) the pK_a of all the protonated clusters in MeCN fall in the relatively narrow range $17.9 < pK_a < 18.9$, irrespective of whether the leaving group is a halide, thiolate or phenolate; demonstrating that protonation occurs at the HOMO of the cluster; that is at the $\{MFe_3S_4\}$ core, rather than the ligands (Henderson and Oglieve 1994a; Grönberg and Henderson, unpublished). Both associative and dissociative substitution pathways can operate after the addition of the proton as shown in Figure 4.

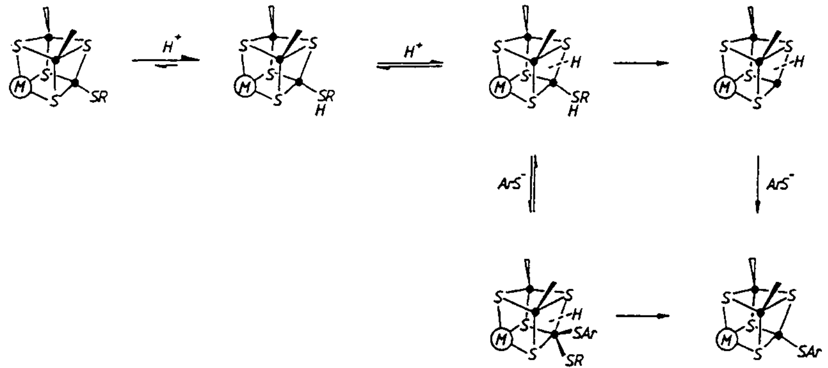

FIGURE 4

There are two general points concerning the reactivity of these clusters which are worthy of further comment.

(1) It must be emphasised that only protonation steps coupled to a substitution reaction can be detected, and any protonation which does not affect the rate of substitution will not be detected. It seems unlikely that the core sulfur atoms are more basic than the terminal thiolate ligands and hence we must conclude that protonation at the thiolato-ligands is not particularly labilising; certainly not as labilising as protonation of the cluster core.

(2) There is an apparent paradox when we compare the labilising forces operating in the non-catalysed associative pathway and the acid-catalysed dissociative pathway. In the latter route the addition of a proton (which will remove electron density from the cluster) labilises the cluster whereas in the former pathway, the addition of a nucleophile (which increases the electron density on the cluster) is labilising.Studies on the non-catalysed substitution reactions of these clusters with $4\text{-}RC_6H_4S^-$ (R = MeO, Me, H or Cl) leads to a rationalisation of this paradox (Henderson and Oglieve, unpublished). The overall rate of substitution does not vary significantly as the para-substituent is changed However, this null effect is due to compensatory effects on the binding constant of the nucleophile and the rate constant for dissociation of the leaving group. The important point is that the leaving group is labilised by electron-withdrawing para-substituents presumably due to the nucleophile and the leaving group competing for the same π-bonding orbitals on the iron. The proton must labilise the cluster using a similar electronic effect.

NEIGHBOURING METAL EFFECTS. Studies with $[Fe_4S_4(SR)_4]^{2-}$ shows that the exclusive pathway for substitution is dissociative, whereas both associative and dissociative pathways operate in the analogous, $[\{MoFe_3S_4(SR)_3\}_2(\mu\text{-}SR)_3]^{3-}$ (Henderson and Oglieve 1993b). Thus the iron centres in molybdenum-containing clusters behave as though they are more electron deficient. It is clearly too naive to ascribe this effect to the molybdenum *per se*, but rather this is just a manifestation of the more general effect that the electron density distribution, and hence the reactivity, throughout the cluster is defined by the components of the cluster.

Recent studies (Henderson and Oglieve, unpublished) on the substitution of the chloro-groups in $[S_2MoS_2FeCl_2]^{2-}$ with $4\text{-}RC_6H_4S^-$ (Figure 5) have demonstrated how the reactivity of a site can be perturbed by changes in the coordination sphere of a neighbouring metal.

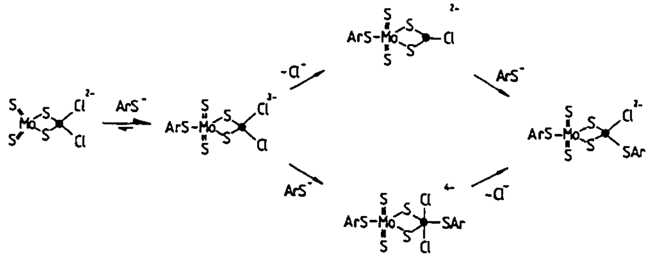

FIGURE 5: ArS = $4\text{-}RC_6H_4S$ (R = MeO, Me, H or Cl)

The important feature in this reaction is that rapid binding of thiolate to the molybdenum means that the reactive species is $[(RC_6H_4S)(S)_2MoS_2FeCl_2]^{3-}$.

Subsequent associative and dissociative substitution pathways at the iron atom are perturbed by the electronic influence of the para-substituent even though it is eight atoms away. The more electron-releasing groups facilitate the dissociative pathway, consistent with the greater electron density imparted to the iron centre. In contrast, electron-withdrawing substituents facilitate the associative pathway but in a slightly less marked manner. The influence of the para-substituent on the rate of both the associative and the dissociative pathways is quantified in the Hammett plot shown in Figure 6. The substitution of the second chloride shows behaviour analogous to that of the first substitution.

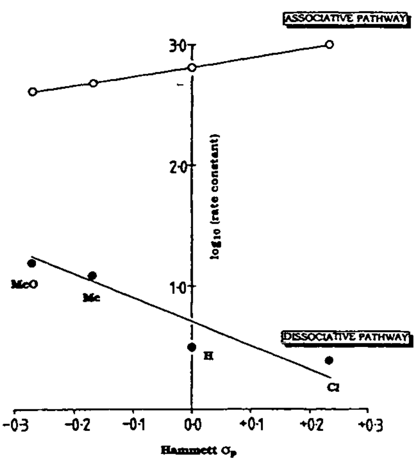

FIGURE 6

That the associative and dissociative pathways respond differently to the electronic influences imposed by a remote site means that reactivity and intimate substitution mechanism of a site within a cluster can be modulated by changes at distant atoms.

Consideration of the behaviour in Figure 6 illustrates that the introduction of more electron-withdrawing ligands at molybdenum inhibits the dissociative pathway of the iron sites, but accelerates the associative pathway.

In the limit the reactivity of a distant site can be switched on or off by subtle electronic changes at a remote metal. This effect is evident in the reactions of $[Fe_2S_2Cl_3(NCMe)]^-$ and thiolate or Br^- (Henderson and Oglieve 1994b). In this system the rate of nucleophilic substitution is inhibited by increasing concentrations of nucleophile. This is a consequence of a mechanism (Figure 7), in which the nucleophile binds preferentially to the substitutionally non-labile iron. This results in a decreased electron density at the labile metal and hence effectively suppresses the loss of the leaving group, MeCN.

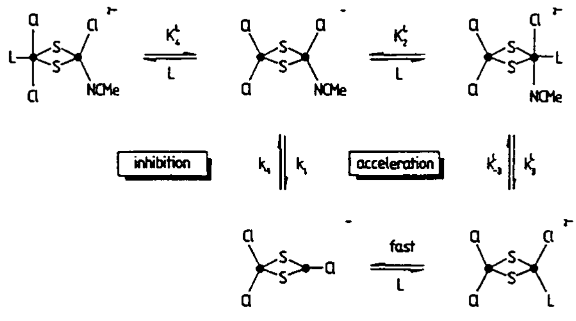

FIGURE 7

Similar behaviour has been observed in the reactions of $[\{MoFe_3S_4Cl_3\}_2(\mu\text{-}SEt)_3]^{3-}$ and $[\{MoFe_3S_4Cl_3\}_2Fe(\mu\text{-}SEt)_6]^{3-}$ (Grönberg and Henderson, unpublished).

INTERACTIONS WITH SMALL MOLECULES. Small molecules such as L = CN^-, N_3^-, halide, CO or N_2O perturb the rate of substitution of $[Fe_4S_4(SEt)_4]^{2-}$ or $[\{MoFe_3S_4(SEt)_3\}_2(\mu\text{-}SEt)_3]^{3-}$ with PhS^- in the presence of $[NHEt_3]^+$ (Henderson and Oglieve, 1993a,b).

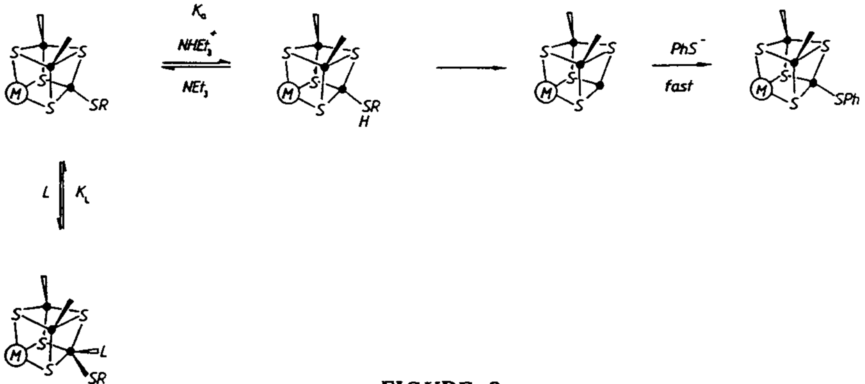

FIGURE 8

This is a consequence of the pathways shown in Figure 8, in which rapid, but short-lived binding of L to the intact cluster competes with the substitution pathway. These interactions have not been identified before because they only have a fleeting existence, and L is not incorporated into the product.

For each L, the binding constant, K_L is larger for the molybdenum-containing cluster. Again this is consistent with the presence of molybdenum imparting a degree of electron-deficiency to the iron sites.

CONCLUSIONS AND SOME SPECULATION. If our contention is correct, that the mechanistic principles highlighted above are so general that they will also operate in the cofactor, then it is worthwhile to speculate a little on the origins of the reactivity of the cofactor. There are three features that may contribute to the reactivity of the cofactor.

(1) Protonation at the iron-sulfur core is a prevalent process at clusters.

Since electrons are also transferred to the cofactor reduction of these cluster-bound protons may be the origin of the enzyme's dihydrogen evolution. In addition, since we have seen that protons labilise cluster ligands, another role for the proton may be to open up a site on the cluster for the substrate to bind.

(2) Perturbing the electron-density at one metal can dramatically effect the reactivity and the intimate mechanism of substitution of a remote metal site.

It is an interesting possibility that by changing the coordination sphere of a metal at one end of the cofactor we can switch on or off reactions at a distant site. It may be that the role for molybdenum or vanadium in the cofactor is to modulate, or fine-tune, the reactivity of the iron centres.

(3) Metal sites within the clusters can bind small molecules either by substitution of a ligand (associative or dissociative activation possible) or by simple binding at the electron-deficient metal centres without substitution.

Recently, it has been argued, based on extended Huckel type calculations, that dinitrogen binds to the three-coordinate iron sites in the cofactor (Deng and Hoffmann 1993; Dance 1994). These calculations must be treated with some caution since: (i) they are based on approximation methods; (ii) the structure of the "active" site is assumed to be that of the crystallographic model of the cofactor and; (iii) the calculations search for energy minima, whereas the coordination mode of dinitrogen which leads to activation may not be a minimum energy geometry. *We must establish experimentally the reactivity of iron-sulfur-based clusters in general, and cofactor specifically, before the intimate mechanism by which dinitrogen is bound and activated by the nitrogenases can be solved finally.*

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Chemistry of N_2H_2 and Other Nitrogen Hydrides Related to N_2 Fixation in the Coordination Sphere of Metal Sulfur Complexes

Dieter Sellmann,
Institut für Anorganische Chemie der Universität, Egerlandstr. 1, D-91058
Erlangen, Germany

The X-ray structural model of the FeMo cofactor (FeMoco) reported by Kim and Rees [1992] yielded the long awaited structural basis, it reconfirmed the importance of sulfur ligands, it told that molybdenum is still a potential binding site of N_2, however, it of course could not specify where the N_2 molecule binds and in that respect shattered the dreams of those who thought all one had to know is the structure of the active center of nitrogenase in order to develop a good catalyst for making cheap fertilizers. This challenge remains and it must be kept in mind that 1) the isolated native FeMoco without its protein does not convert N_2, 2) the isolated FeMoco is a relatively short-lived species in aqueous media (K. B. Burgess 1990) and 3) thermodynamics state that the reaction $N_2 + 6\ H^+ + 6\ e^- \longrightarrow 2\ NH_3$ is exoenergetic needing a potential of only -280 mV at pH=7 which is less than is needed to reduce protons to dihydrogen (-410 mV at pH=7). In particular this "mild" redox potential eliminates all those model systems as potentially competitive catalysts which need at some stage strongly reducing reagents such as alkali metals in order to bind and to reduce N_2.

Our attempts to tackle this challenge is to combine transition metals and sulfur ligands like nature, try to isolate potential intermediates in order to learn about mechanisms and to find perhaps on this way a catalyst which allows to reduce the drastic conditions of the Haber-Bosch process and to reach the limits set by thermodynamics.
Metal sulfur entities exhibit a couple of characteristic features which might be essential for the reduction of N_2 by addition of six protons and six electrons. Transition metals coordinated by thioether, thiolate and/or sulfide sulfur donors frequently show redox activity, Brönsted acid-base properties and vacant sites at which small molecules can be coordinated, activated and/or stabilized (D. Sellmann et al. 1994a).

All of the nitrogen hydrides N_2H, N_2H_2, N_2H_3, N_2H_4, $N_2H_5^+$, NH, NH_2 are of interest when N_2 is reduced to NH_3 in the coordination sphere of metal sulfur complexes. The majority of these species is highly unstable or even unknown in free state, but some of them can become remarkably stabilized by coordination to [MS] centers.

Scheme 1 shows NH_2 and N_2H_3 molybdenum sulfur complexes which form upon reaction of a molybdenum chloro complex with either NH_3 or N_2H_4.

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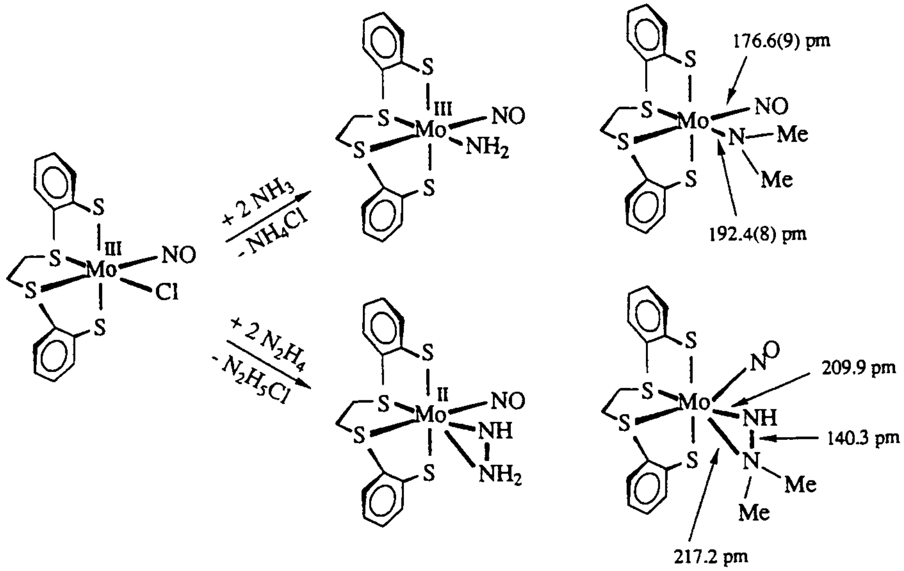
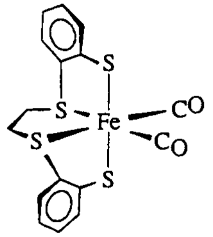
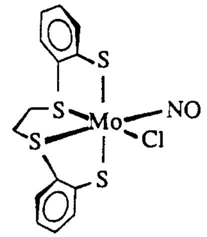
Scheme 1: Coordination and stabilization of NH_2 and N_2H_3 at [MoS] centers



The dimethyl derivatives [Mo[NMe_2](NO)('S_4')] (D. Sellmann et al. 1989) and [Mo($NHNMe_2$)(NO)('S_4')] (D. Sellmann et al. 1991) were characterized by X-ray structure determination, and while substitution of Cl by NH_2 leaves unchanged the Mo oxidation state, substitution by N_2H_3 leads to a change from Mo(III) to Mo(II). This can be concluded from the ^{95}Mo NMR shifts of [Mo(N_2H_3)(NO)('S_4')] (-444 ppm) and [Mo(NMe_2)(NO)('S_4')] (+340 ppm). N_2H_3 complexes are rare species (R.R. Schrock et al. 1988, G. Huttner et al. 1991), and [Mo(N_2H_3)(NO)('S_4')] is in fact the single example in which N_2H_3 is coordinated to a [MS] center. More important, however, is the fact that [Mo(N_2H_3)(NO)('S_4')] is isoelectronic with [Mo(NH_2O)(NO)('S_4')] which readily forms in a $2H^+/2e$ reduction from [$Mo(NO)_2$('S_4')] and $NaBH_4$ in MeOH (D. Sellmann et al. 1992a). The easiness of this reduction can be traced back to the simultaneous transfer of two electrons and two protons such that unfavourable 19 VE or 20 VE intermediates are avoided, and for that reason the hypothetical N_2 complex [Mo(N_2)(NO)('S_4')]$^-$ being isoelectronic with [$Mo(NO)_2$('S_4')] becomes a particularly attractive target species.

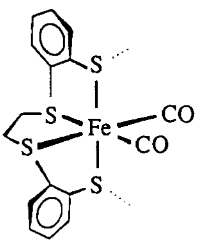
The important coupling of transfer of protons and electrons was the reason to investigate the question in which way protonation or isoelectronic alkylation of thiolate donors influence the electron density at the metal centers, redox potentials and metal ligands bonds in complexes such as [$Fe(CO)_2$('S_4')] and [Mo(NO)(Cl)('S_4')].





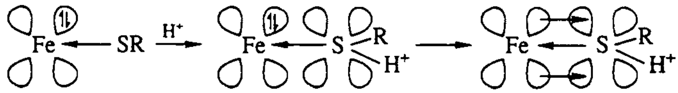
Protonation takes place stepwise and reversibly at the thiolate donors, yields labile thiol complexes and shifts the ν(CO) and ν(NO) bands by some +40 cm^{-1} per added proton indicating a remarkably strong decrease of electron density at the Fe and Mo centers. Alkylation takes place in a similar way, causes nearly identical ν(CO) and ν(NO) shifts, but is irreversible such that the resultant complexes could be isolated and characterized by X-ray structure determination. The strong shift of the ν(CO) bands to higher wavenumbers in the series of $[Fe(CO)_2('S_4')]$ and its alkylated derivatives, which is comparable to the ν(CO) shift in the homoleptic CO complexes $[Mn(CO)_6]^+$ (2090 cm^{-1}) and $[Cr(CO)_6]$ (2000 cm^{-1}), suggested a correlation between ν(CO) frequencies and bond distances . As outlined in Scheme 2 and in contrast to expectations, such a correlation does not exist. Despite of the ν(CO) shifts, the average FeS bond distances remain invariant, and in addition it is to be noted that in this series Fe-S(thiolate) and Fe-S(thioether) distances are practically identical (D. Sellmann et al. 1994b).

Scheme 2: ν(CO) frequencies and FeS distances - Bonding features



	$\bar{\nu}$(CO)cm^{-1}	$\bar{d}$(Fe-S) pm
$[Fe(CO)_2('S_4')]$	2010	228.4(3)
$[Fe(CO)_2(Et\text{-}'S_4')]^+$	2049	228.9(2)
$[Fe(CO)_2(Et_2\text{-}'S_4')]^{2+}$	2091	228.1(2)

- ν(CO) indicate (strong) decrease of electron density at Fe
- FeS distances remain invariant
- alkylations (and protonations) of thiolate donors switch on $\overset{\frown}{Fe - S}$ π-back bonds

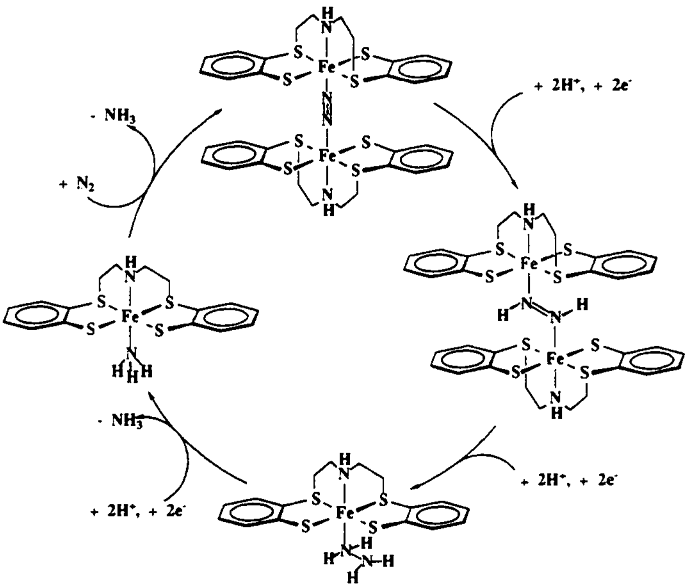


The invariance of the Fe-S distances can be traced back to the special features of metal sulfur bonds. In the neutral $[Fe(CO)_2('S_4')]$ the Fe-S(thiolate) bonds basically have single bond character. Protonation or alkylation of the thiolate donors weakens the σ bonds, but simultaneously the sulfur donors gain π acceptor properties. Weakening the σ donor bond and formation of a π acceptor bond compensate each other such that the FeS distances stay unchanged although the electron density at the Fe center, according to the ν(CO) frequencies, undoubtedly decreases.

Similar results were obtained with $[Mo(NO)Cl('S_4')]$ and $[Mo(NO)Cl(Me\text{-}'S_4')]BF_4$ (D. Sellmann et al. 1995). This pair yielded cyclic voltammograms showing that the decrease of electron density at the Mo center causes a positive shift of the potential of corresponding redox steps by 440 mV. In conclusion, and when alkylation of thiolate donors is regarded as kind of 'frozen-in' protonation, it can be stated that protonation (alkylation) facilitates the reduction of metal sulfur complexes by some 500 mV. These results are relevant for the requirements which have to be fulfilled in order to achieve the first $2H^+/2e$ reduction step of N_2 complexes. This step is considered by others (A.E. Shilov et al. 1970,1977) and ourselves (D. Sellmann 1972) to be the most difficult and consequently a key step when N_2 is to be reduced down to NH_3. While Shilov et al. favour 4e redox steps yielding hydrazine(derivatives) in order to circumvent the diazene

stage, we regard N_2H_2, which is extremely unstable in free state ($\Delta H_f = +212$ kJ/mol), a viable candidate because it can become highly stabilized in complexed state such that the principles of catalysis are met stating, inter alia, that complexation entirely changes the properties, in particular the stability and reactivity, of molecules.
This is illustrated by the model cycle for the reduction of N_2 in the coordination sphere of [FeS] complexes in Scheme 3 (D. Sellmann et al. 1992b).

Scheme 3: Model cycle of N_2 fixation catalyzed by [Fe('NHS$_4$')] fragments



The N_2H_2, N_2H_4 and NH_3 complexes of this cycle could be isolated and completely characterized. It is further to be noted that the cycle exhibits no unusual Fe oxidation states (Fe(0) or Fe(I)) but only Fe(II) complexes, these, however, are either low-spin (N_2H_2) or high-spin (N_2H_4 and NH_3) such that the two changes of spin-state have to be envisaged when going through the cycle. The spectroscopic properties and the structure (Fig. 1) of the N_2H_2 complex allowed to recognize three important factors (D. Sellmann et al. 1992b) that stabilize the trans-diazene ligand ($d_{NN} = 130.0(7)$ pm).

1) Electronic stabilization by a 4c-6e π-system in the Fe$\doteq$N$\doteq$N$\doteq$Fe unit might be the most important factor. 2) Steric shielding of the N_2H_2 ligand by the bulky [Fe('NHS$_4$')] fragments adds kinetic stabilization. 3) Specifically due to the Lewis base properties of the thiolate donors, N-H···S bridges exist which, by a very cautious estimate, yield ca. 70 kJ bond energy that neutralize at least one third of the positive ΔH_f of N_2H_2 in free state (D. Sellmann et al. 1992b).

[μ-N_2H_2{Fe('NHS$_4$')}$_2$] is obtained in low yields only and exhibits insufficient solubility in order to investigate its chemistry in homogeneous phase. These set-backs could be overcome by synthesis of analogous [μ-N_2H_2{Fe(PR$_3$)('S$_4$')}$_2$] complexes with R = Pr, Bu (D. Sellmann et al. 1994c). They exhibit the identical structural and stabilizing features as [μ-N_2H_2{Fe('NHS$_4$')}$_2$] and differ from this complex only with respect to substitution of the NH donor by PR$_3$, however, they form in high yields and are so well soluble that cyclic voltammograms (CV's) could be obtained (Fig. 2).

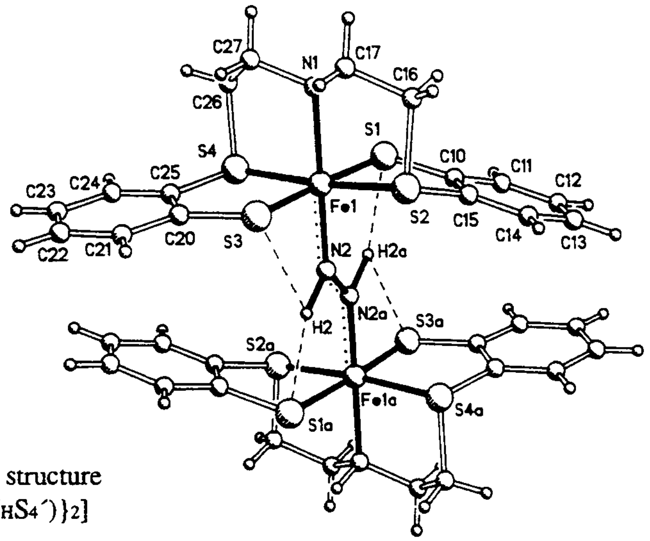
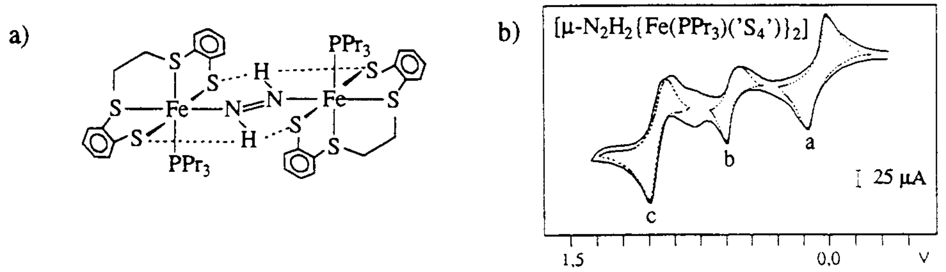


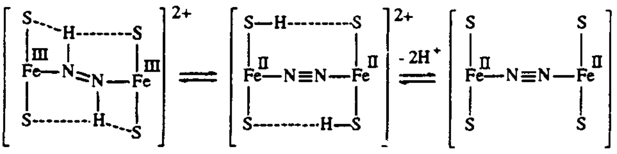
Fig. 1: Molecular structure of $[\mu\text{-}N_2H_2\{Fe('N_HS_4')\}_2]$



c) $$[\overset{II}{Fe}\text{-NH=NH-}\overset{II}{Fe}] \overset{a}{\rightleftharpoons} [\overset{III}{Fe}\text{-NH=NH-}\overset{II}{Fe}]^{+} \overset{b}{\rightleftharpoons} [\overset{III}{Fe}\text{-NH=NH-}\overset{III}{Fe}]^{2+}$$

Fig. 2: a) Schematic drawing of the molecular structure as determined by X-ray structure analysis, and b) cyclic voltammogram of $[\mu\text{-}N_2H_2\{Fe(PPr_3)('S_4')\}_2]$ (in CH_2Cl_2, potentials vs. NHE). c) Assignment of Fe oxidation states in resultant redox species.

The CV exhibits three reversible anodic redox waves yielding the mono-, di- and tris-cations, and assignment of formal Fe oxidation states shows that the dication exhibits two Fe(III) centers. In conjunction with the effects of protonation discussed above and familiar intramolecular electron transfer reactions between metal centers and ligands, this dication could gain special significance with respect to the conditions that have to be met in order to reduce dinitrogen complexes under mild conditions,i.e., at redox potentials not very different from -280 mV. Scheme 4 shows drawings of the central unit of the dication considering only the relevant thiolate donors (of the 'S4' ligands), the two Fe centers and the N_2H_2 ligand.



Scheme 4: Schematic representation of redox isomerism and deprotonation of the [μ-$N_2H_2\{Fe(PR_3)('S_4')\}_2]^{2+}$ dication

Dissociation of the N-H bonds and intramolecular electron transfer could lead to a twofold protonated binuclear Fe(II)-N_2-Fe(II) complex which is a redox isomer of the diazene complex dication. Subsequent deprotonation could give the neutral N_2 complex. Such a sequence of reactions, the principle of microscopic reversibility, the reversibility of the redox waves in the CV, and the above discussed influence of protons upon redox potentials finally allow to hypothesize that the as yet not isolable N_2 complex first has to be protonated and may also become stabilized by such a protonation, before it can be reduced by two electrons, either via the redox isomeric dicationic Fe(III) diazene complex or directly, to give the neutral diazene complex.

In conclusion, the study of fully characterized complexes with nitrogen hydride ligands being potential intermediates of N_2 reduction can reveal pathways which may yield a solution for the challenge to chemically reduce N_2 under as mild conditions as nitrogenases and which simultaneously give clues to the mechanisms of N_2 fixation taking place at the [FeMoS] centers of these enzymes.

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THE CHEMICAL MECHANISM OF BIOLOGICAL NITROGEN FIXATION AND THE CHEMISTRY OF MODEL SYSTEMS

LEIGH, G.J.
School of Chemistry and Molecular Sciences, University of Sussex,
Brighton, BN1 9QJ UK

Since the discovery of the first dinitrogen complex (Allen,Senoff, 1965), it has been generally assumed that the chemistry arising from such systems would enable us to understand the chemistry of nitrogenase, if not sooner then later, and certainly once the structure of nitrogenase had been established. This assumption has been upset by two related sets of events. The first was the discovery that there is more than one kind of nitrogenase (Eady, Leigh, 1994). This caused us to wonder whether molybdenum really is at the active site of conventional nitrogenases. It would appear that vanadium can replace molybdenum in some of its functions, and that iron may be the active metal in all three kinds of nitrogenase recognised hereto. The second was the elucidation of the structures of two different nitrogenase molybdenum-iron (MoFe) proteins (Kim, Rees, 1992; Bolin *et al.*, 1993), which are supposed to contain the active sites for nitrogen fixation. The molybdenum atoms seem coordinatively saturated, and apparently excluded from reaction with dinitrogen, but there is no other obvious active site.

The discussions on mechanism that have followed these discoveries have been marked by considerable ingenuity and a lot of imagination. Yet it seems unlikely that the chemistry revealed to us by dinitrogen is entirely unrelated to the chemistry of nitrogenase. It may well be true that N_2 can be caged within a metal cluster, or that it can insert into a metal-metal bond, or even that it can bind to the face of a metal cluster. However, as long as there are no well established examples of such reactivities, it is probably unwise to postulate them for nitrogenase function with too much enthusiasm. In addition, we know a great deal about the biochemistry of dinitrogen reduction, and it is therefore unwise, and possibly misleading, to ignore that data, and the data we have on alternative-substrate reduction. This review attempts to reconcile both biological and chemical information in a picture of dinitrogen reduction which is consistent with both sets of data, and does not extrapolate too much from established facts.

If dinitrogen really does bind to a metal ion in nitrogenases, then it must do so to iron, vanadium, or to molybdenum, or to a combination of these. The formal oxidation states to which dinitrogen binds in model compounds are iron(II), iron(0), vanadium(III), vanadium(II), vanadium(-I), molybdenum(IV), molybdenum(II), and molybdenum(0)

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(Leigh, 1995). What we know about the relevant dinitrogen chemistry of these complexes can be summarised as follows. Binding of dinitrogen causes a lengthening of the N-N bond. In many cases, and especially when the binding is at one end only, the lengthening is only marginal, perhaps less than 0,1Å. However, there is no linear relationship between the length of the N-N bond, the strength of the binding to the metal, the N-N stretching frequency, $\nu(N{=}N)$, and the degree of activation to protonation (Deeth,Field, 1994; Deeth,Langford, 1995). Even in cases where there is activation to protonation, none of the quoted parameters give a clue as to the ultimate products of dinitrogen protonation. In the case of $[[\{(Pr^i{}_2PCH_2SiMe_2)_2N\}ZrCl]_2(N_2)]$ the N-N separation is 1,548(7)Å, so that one might be tempted to suppose that there is no N-N bond at all. In fact, protonation yields hydrazine (Fryzuk *et al.*, 1990).

The two major classes of dinitrogen complex have dinitrogen bound singly at one end,or bound at both ends, bridging between two metal ions, usually of the same kind and in the same formal oxidation state (Leigh, 1994). The iron(0) compounds fall into both classes, but the iron(II) compounds are generally singly-bound. Vanadium-(II) and -(III) complexes are only bridging, whereas the the vanadium(-I) complexes are singly bound. Finally, molybdenum-(0) and -(II) compounds have N_2 singly bound, whereas molybdenum(IV) compounds always contain N_2 bridges. Higher oxidation states seem to provoke bridging, and one can rationalise this by arguing that a single ion in a high oxidation state cannot provide enough π-electron density to stabilise binding at one end only. If the oxidation state is too high overall, then N_2 is simply not bound . This is like the phenomenon that is observed with carbonyls. It seems unlikely that molybdenum(0) or vanadium(-I) would be attainable in biological systems, so these oxidation states are not considered further here.

However, this implies that only molybdenum(II) and some iron complexes are reasonable candidates for binding dinitrogen. Vanadium is excluded because it requires too great a degree of reduction, or because it would otherwise form only bridging complexes. The remarkable splitting of the N-N triple bond by $[Mo\{NBu^t(C_6H_3Me_2)\}_3]$ to form a nitrido complex (Laplaza,Cummins, 1995) is likewise unlikely to be copied by nitrogenase. However, we predict that vanadium(I) complexes will eventually be synthesised, and these might then be suitable candidates.

The next question to consider is whether the remaining complexes actually show any chemistry that would be feasible in nitrogenase.The stepwise protonation of dinitrogen bound to molybdenum(0) has been well-established (Henderson *et al.*, 1983). Intermediates have been isolated from a series of different reactions, corresponding to the moieties MoNNH, $MoNNH_2$, and $MoNNH_3$. The mechanisms of the corresponding reactions have also been established by kinetic studies (Henderson, 1982). In none of these cases has a unique mechanism for the fission of the N-N bond been determined. However, for some tungsten compounds ammonia has been generated concurrently with complete degradation of the complexes (Chatt *et al.*, 1975).Where it has proved possible to reduce appropriate reaction mixtures, either chemically (Hussain *et al.*, 1982) or electrochemically (Pickett,Talarmin, 1985), then cyclic systems have been developed, though principally with tungsten rather than with its congener, molybdenum. This combined protonation/reduction is presumably more akin to what occurs at the active site of nitrogenases.

The site of the third protonation has been the matter of speculation. Despite the fact that it was shown some years ago that protonation of coordinated N_2 seems to lead to NNH_3 rather than to $NHNH_2$ (Galindo et al., 1987; Barclay et al., 1990), this has not prevented this fact being reported yet again (Wagenknecht,Norton, 1995). The reason that this route is adopted is not hard to find. The anion NH_2NH^- undergoes a redox disproportionation whenever a kinetic route is available, and such disproportionations have been observed in complexes synthesised both from hydrazide(1-) (Latham,Leigh, 1986) and from dinitrogen (Bossard *et al.*, 1982). Evidently, $NHNH_2^-$ is thermodynamically unstable, as is hydrazine itself.

Dinitrogen can bind to metal sites of many different kinds. It can displace a variety of other ligands, including ammonia, water, chloride, and dihydrogen. It is also capable of binding to empty sites of the right kind (Leigh, 1995). The most important recent discovery in this area is the recognition that N_2 can also displace carboxylate groups from coordination with tungsten (Hughes *et al.*, 1994). This may explain how N_2 can bind to the apparently coordinatively saturated molybdenum in nitrogenases. The loss of H_2 from the compounds described here is subsequent to the binding of N_2, and the significance of this will become evident in the discussion below. The displacement of H_2 from complexes by N_2 has been used earlier to explain the stoichiometry of reduction of N_2 by Mo-Fe nitrogenases, where 8 electrons are required and at least two are apparently "wasted" as dihydrogen. It has also been used to explain the formation of HD during N_2 reduction in the presence of D_2 (Chatt, 1980). The model for this phenomenon requires that at least 3 electrons reach the active site before N_2 binding occurs, which is nicely consistent with the Lowe-Thorneley kinetic model for nitrogenase function (Thorneley,Lowe, 1984). However, it is not necessary that all the electrons are consumed at the same metal site, though this is generally assumed to be the case. There is a further complication in that displacement of H_2 by N_2 in model complexes generally occurs by prior loss of dihydrogen. In that case, it is not clear why HD formation requires that N_2 be also reduced at the same time.In fact, no model so far promulgated has explained this satisfactorily, and no model explains why the dihydrogen-evolving stoichiometries of the other two nitrogenases are so different from that of the Mo-Fe nitrogenase.

It is clear that it is unwise to assume that all the reactivity observed in nitrogenases is occurring at a single metal site, or that that site is necessarily the correspondig one in all three nitrogenases. Vanadium may well take the place of molybdenum in the Mo-Fe nitrogenase, and there is a lot of circumstantial evidence to suggest that it does (Eady, 1991). The two elements are in a diagonal relationship in the Periodic Table, which makes this even more plausible. However, iron is in no such relationship with either of these elements, which makes it unlikely that in the third nitrogenase it simply fills the equivalent position to the other two elements. In fact, the reactivities of the third nitrogenase are so different from those of the other two that it is easy to accept that it does not. This then poses two questions. Is there chemistry to suggest that iron can also mediate dinitrogen reduction? Is there evidence from substrate reduction that more than one redox site may be active under certain circumstances? The "active site" is not a single atom, but a complex and hitherto unrecognised metal-sulfur cluster, and multi-position activity is not unreasonable.

It has been long recognised that molybdenum in complexes is capable of mediating the reduction of dinitrogen to give ammonia. The dinitrogen is bound to molybdenum at one end only, and this kind of binding in nitrogenase is not excluded by the published nitrogenase structures. As discussed above, the chemistry of vanadium does not suggest that vanadium alone constitutes the active site. Until recently (Leigh, Jimenez-Tenorio, 1991), there was no chemistry of iron that would imply that iron might also constitute the active site. However, there are now several cyclic systems, typified by the following example, that show that iron is indeed a candidate:

$[FeCl_2(dmpe)_2]$ ---> $[FeH_3(dmpe)_2]^+$ ---> $[FeH(N_2)(dmpe)_2]^+$ ---> $[Fe(N_2)(dmpe)_2]$ (?) ---> $[FeCl_2(dmpe)_2] + H_2 + NH_3$.
Dmpe is $Me_2PCH_2CH_2PMe_2$.

The crucial step is the third, in which an iron(II) compound is converted to an iron(O) compound simply by addition of base. The loss of the proton from the iron releases two electrons previously localised in the iron-hydrogen bond, and this kind of process is quite feasible in an enzyme. The yields of ammonia are quite low, but the chemistry behind the reactions is very thought-provoking.

Chemistry can tell us little, as yet, about systems in which N_2 bridges between iron, and iron, vanadium, or molybdenum.

The most interesting substrates of the nitrogenases, apart from N_2, are probably hydrocarbons. The reduction of acetylene, generally to give ethylene, and stereochemically *cis* (Dilworth, 1966) has been recognised for many years. The interesting question is not the stereochemistry, which is not at all uncommon in such reductions, but the fact that the reduction essentially stops at ethylene and does not procede to ethane.Cyclopropene is also reduced, to cyclopropane, stereochemically *cis* , and to propene and C-F bonds in 3,3-difluorocyclopropene are cleaved. There is evidence that metal-hydrogen bonds are concerned in the reduction, and that hydrogen-transfers as well as simple hydrogenations of double and single bonds are involved (McKenna et al., 1980, 1981).

Fundamental studies of the protonation of coordinated alkynes have been reported recently (Henderson *et al.*, 1995). For example, the treatment of $[V(Cp)_2(PhCCPh)]$ with HCl yields *cis*-PhCHCHPh (85%) and *trans*-PhCHCHPh (15%). Mechanistic analysis suggests that the *cis*-compound is the result of initial formation of a metal hydride followed by migration of the hydride to give a *cis*-vinyl complex. Direct protonation at the alkyne yields the *trans*-vinyl species. Other work (Henderson, Oglieve, 1994) suggests that the product of protonation of coordinated allyl with a large excess of acid yields propyne, whereas with lower concentrations of acid propene is formed. Prior protonation at the metal favours the former, and at the hydrocarbon favours the latter. Whatever the situation in nitrogenases, this suggests that the properties of hydrocarbon reduction exhibited by nitrogenase are founded in fundamental metal-carbon chemistry rather than in peculiar properties of nitrogenases.

As discussed above, the proposition that formation of a trihydride explains the generation of HD during dinitrogen reduction under D_2 suffers from the fact that since the replacement of H_2 by N_2 within the coordination sphere of a metal polyhydride is normally a dissociative process, then it is not clear why N_2 should be a necessary requirement for HD formation. It should equally proceed in the absence of N_2. However, if N_2 displaces not H_2 but another ligand, then this problem disappears. The evolution of H_2 can then be seen as the consequence of dinitrogen-binding, and the trihydride postulate, though it may still be justified, is not strictly relevant. As has recently been shown (Hughes *et al.*, 1994), the following series of reactions can occur:

$[WH(OOCCH_3)(dppe)_2]$ ---> $[WH_2(OOCCH_3)(dppe)_2]^+$ ---> $[WH_2(OOCCH_3)(dppe)_2]^-$ ---> $[WH_2(N_2)(dppe)_2]$ + $(OOCCH_3)^-$, dppe = $Ph_2CH_2CH_2PPh_2$. Subsequent reaction with N_2 produces a tetrahydride, and a similar series of reactions occurs with Mo. This shows not only that a carboxylate can be replaced by N_2, but that H_2 evolution can follow N_2 uptake. The significance of this will be evident later.We now consider the principal clusters in the two examples of Mo-Fe protein for which the structures have been established. Their probable participation in the reduction process has been discussed, but the chemistry at the atomic level is still unclear. Apparently the Fe_8S_n clusters, of structure not completely resolved, receive electrons from the smaller proteins, which contain conventional Fe_4S_4 clusters. They then pass them on to the larger cluster, which also contains the site at which N_2 is bound and reduced (Dean *et al.*, 1993). This cluster may be regarded as containing 6 three-coordinate iron(II) sites, a four-coordinate iron(II) site, and a six-coordinate molybdenum(IV) site. The overall charge is not clear, but the oxidation level of the proteins of which the structures have been determined is not reactive with N_2. The three-coordinate irons are very unusual, and the molybdenum appears coordinatively saturated, if not electronically saturated. The large cluster does not reduce substrates when removed from its protein, but the MoFe protein does react with H_2, which it can use an electron source in place of the smaller Fe protein.

It is not obvious where N_2 might react in this assembly of clusters. Various suggestions have been made, including:insertion into the void in the large cluster (Kahlal *et al.*, 1991), unlikely because the void is probably too small; interaction at positions occupied by the sulfur atoms in the central belt around the larger cluster (Deng,Hofmann, 1993), based partly upon inaccurate preliminary data, but in any case without real precedent; and interaction with four iron atoms on a face of the larger cluster, which does not have cylindrical symmetry because of the coordination shell of the molybdenum and the geometry and constitution of the protein chain (Dance, 1994), although such metal cluster-N_2 interactions have no precedent in molecular chemistry.
A further criticsm that one might make of many such discussions is that they are often concerned with the binding of N_2 to a given site, and its consequent "activation". However,they really concern "potentiation" to reaction more than activation. For reactivity electrons are also required, and the reason that vanadium(III), for example, binds N_2 but does not mediate its reduction, whereas vanadium(II) does both, is that the V^{III}/V^{II} couple can reduce N_2 whereas the V^{IV}/V^{III} couple cannot under the circumstances usually prevailing.

We now consider a possible mechanism of N_2 reduction on the basis of established chemistry. First we note that the formation of HD by nitrogenase, but only in the presence of N_2, requires that the H and the D in HD come from different sources, because the deuterium never passes into solution. It has also been shown that the comparable reduction of N_2 in the presence of HD does not generate D_2. Since the D necessarily comes from the gas phase, all the H must arise from solution. This is consonant with the observed reactivity of the MoFe protein with H_2. We postulate that molybdenum is the likely binding site of the N_2, and that the binding is achieved by displacement of a carboxylate of the homocitrate, which, however, remains tethered to the molybdenum *via* its alkoxide link. The three-coordinate iron atoms can form iron-hydrogen bonds, either by reaction with protons plus electrons (the latter from the Fe_8S_n cluster) or, if the system is reduced sufficiently, by reaction with H_2. Such bonds to hydrogen can act as stores for electrons, to be released by a change in pH which could be controlled by the enzyme, but apparently the requisite level of reduction to bind H_2 can only be reached if N_2 is also bound at molybdenum.The evolution of H_2 from iron could occur as follows.

$$Fe^{II} + H^+ + e \text{--->} Fe^{III}H$$
$$Fe^{III}H + H^+ + e \text{--->} Fe^{II} + H_2$$

An alternative reaction can be envisaged, if the system is electron-rich enough.

$$2Fe^{III}H + H_2 \text{--->} 2Fe^{IV}H_2 \text{--->} 2Fe^{II} + 2H_2$$

Such a reaction with D_2 rather than H_2 provides a mechanism for HD formation and explains why HD cannot give rise to D_2.

The overall binding reaction of N_2 can be represented by the following equation.

$$N_2 + Mo(OOCR) + 2Fe^{III}H \text{--->} MoN_2{}^{+} + (OOCR)^- + 2Fe^{II} + H_2$$

The iron is included in this equation simply to maintain the H_2 evolution when N_2 binds, and is not really required, but it could also reduce the molybdenum. Finally, one can regard the protonation of the coordinated N_2 to be coupled to electron release from iron in the following formal terms.

$$2Fe^{III}H \text{--->} 2Fe^{I} + 2H^+$$
$$MoN_2 + 2Fe^{I} + 2H^+ \text{--->} 2Fe^{II} + MoNNH_2\text{, etc}$$

The chemistry which would then follow on molybdenum is well established dinitrogen chemistry.

The basic innovation that we are suggesting is that the mechanism of nitrogen fixation in biological systems is a multi-site process. It is unlikely that Nature would have evolved such a complex system unless it were necessary. It is easy to accept that, in the vanadium nitrogenases, vanadium simply takes the place of molybdenum. The mechanism of N_2 reduction would be similar, but with a different balance of proton and dinitrogen reduction due to different redox potentials. Finally, because it seems unreasonable to postulate that iron is capable of replacing either of the other two metals and of retaining analogous fixation activity, it is possible that the FeS core of the Mo-Fe cluster is capable itself of interacting weakly with N_2 and reducing it inefficiently. This is likely to have little significance in the functioning of whole organisms and the third nitrogenase may not be so much a real nitrogenase as an incomplete enzyme that retains some residual nitrogenase properties.

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CATALYTIC REDUCTION OF HYDRAZINE AND ACETYLENE TO AMMONIA AND ETHYLENE AND STOICHIOMETRIC REDUCTION OF CN$^-$ TO AMMONIA AND METHANE BY FE/M/S CLUSTERS (M =MO, V) WITH STRUCTURAL FEATURES SIMILAR TO THOSE OF THE FE/MO/S SITE IN NITROGENASE

COUCOUVANIS, D., MOSIER, P.E., MALINAK, S., LAUGHLIN, L., AND DEMADIS. K.D.
Department of Chemistry, University of Michigan, Ann Arbor , MI 48109, USA

Recent single crystal X-ray structure determinations of the Fe-Mo protein of nitrogenase from *Clostridium pasteurianum* and *Azotobacter vinelandii* (Kim, Rees, 1992a,b; Chan et al., 1993; Bolin et al, 1993) have revealed the structure of the Fe/Mo/S aggregate to near atomic resolution. The Fe/Mo/S cluster consists of two cuboidal subunits, Fe_4S_3 and $MoFe_3S_3$, bridged by three S^{2-} ions and is anchored on the protein matrix by a Fe-coordinated cysteinyl residue and by a Mo-coordinated imidazole group from a histidine residue. The Mo atom also is coordinated by a homocitrate molecule that serves as a bidentate chelate. Unusual structural features include the unprecedented trigonal planar coordination geometry for the six μ-S-bridged iron atoms, and the unusually short Fe-Fe distances across the two subunits (2.5-2.6A). The location of the Mo atom at the periphery of the $MoFe_7S_{8-9}$ cluster was unexpected and was interpreted as an indication that the Mo atom may not be directly involved in N_2 fixation. The clearly defined structural features of the nitrogenase Fe/Mo/S center have not led to a clear understanding of the mode of substrate activation and reduction. Indeed many questions regarding the catalytic function of nitrogenase remain unanswered. One of these questions concerns the role of the Mo atom (and of the V atom in an alternate nitrogenase) in the function of the M centers in nitrogenase. The latter may be examined by reactivity studies on appropriate model complexes.

The models that we have chosen to examine in substrate reduction studies contain as a commom structural feature the MFe_3S_4 "cubane" unit (M = Mo, V) and their chemistry has been the subject of several detailed reviews (Coucovanis, 1981; Holm, Simhom, 1985; Kovacs, Holm, 1987a,b; Ciurli, Holm, 1989). These are a) the single cubane (SC) clusters ($[(L)MoFe_3S_4Cl_3(CH_3CN)]^{n-}$; L= Cl_4-catecholate,n = 2; L = citrate, n = 3); b) the doubly-bridged double-cubanes (DBDC), $\{[(Cl_4\text{cat})MoFe_3S_4Cl_2]_2(\mu\text{-}NH_2NH_2)(\mu\text{-}S)]\}^{4-}$; and c) the singly-bridged double-cubane (SBDC) clusters, $[(Cl_4\text{cat})MoFe_3S_4Cl_3]_2(\mu\text{-}NH_2NH_2)]^{4-}$. The MFe_3S_4 cores in these model complexes are structurally quite similar to the MFe_3S_3 cuboidal subunits in the nitrogenase cofactor.

Catalytic disproportionation and reduction of hydrazine.

The $[(L)MoFe_3S_4Cl_3(CH_3CN)]^{n-}$ (SC) clusters have been found effective in the catalytic reduction of hydrazine, N_2H_4, (a nitrogenase substrate) to ammonia in the presence of cobaltocene as a source of electrons and lutidine hydrochloride as a source of protons (Table 1; Coucouvanis et al, 1993). Reactivity studies have shown that the Mo-

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coordinated terminal ligands have a profound effect on the catalytic properties of the $MoFe_3S_4$ cubanes and this result strongly implicates the Mo atom as the site directly involved in the reduction of N_2H_4. In contrast, a low level of reactivity is observed in attempted reductions of N_2H_4 by the $[(Cl_4\text{-}cat)MoFe_3S_4Cl_3]_2(\mu\text{-}NH_2NH_2)]^{4-}$ and $\{[(Cl_4\text{-}cat)MoFe_3S_4Cl_2]_2(\mu\text{-}NH_2NH_2)(\mu\text{-}S)]\}^{4-}$ singly and doubly-bridged double cubane clusters and no reactivity at all is detected with the all-iron, $[Fe_4S_4Cl_4]^{2-}$ cubane. The inability of the $\mu\text{-}NH_2NH_2$ molecules in the SBDC and DBDC to undergo reduction to ammonia indicates that the reduction requires a free lone pair of electrons on the hydrazine molecule that is needed for an initial protonation step. Isolation and characterization of the $[(Cl_4\text{-}cat)MoFe_3S_4Cl_3(N_2H_5)]^-$ cluster and the subsequent reduction of the $N_2H_5^+$ ligand to ammonia support the contention that protonation of the substrate precedes reduction which is best carried out on a single metal site. The Mo-bound tridentate citrate ligand, in the citrate-cubane catalyst, rather than blocking access of hydrazine (and inhibiting subsequent reduction) promotes the catalytic process.

Table 1. Catalytic reduction of hydrazine by $[(Cl_4\text{-}cat)MoFe_3S_4Cl_3(CH_3CN)]^{2-}$ in CH_3CN in the presence of $Co(Cp)_2$ and Lut.-HCl as sources of e^- and H^+, respectively.

N_2H_4/Catalyst[a]	time (min)	NH_3[b,c]	% reduction[e]
10[d]	30	20.0	100
10	30	13.2(7.0)	66
	60	14.6(1.1)	73
20	30	32.8(1.5)	82
	60	37.2(1.7)	93
40	30	61.6(2.5)	77
	60	69.6(3.1)	87
100	30	60.0(4.0)	30
	60	68.0	34
	120	104.0	52

a) Concentration of catalyst is 1×10^{-4}M. b) Ammonia reported as equivalents produced per equivalent of catalyst, quantified by the indophenol method. c) Numbers in parentheses represent the standard deviation from the mean when more than three measurements were made. d) Results obtained with the $[(citrate)MoFe_3S_4Cl_3]^{3-}$ catalyst. e) At ambient temperature.

One can speculate that protonation of the citrate ligand causes dissociation of one of the oxygen donor atoms and provides the N_2H_4 molecule access to the Mo coordination sphere. The availability of the added proton on the citrate ligand conveniently facilitates intramolecular transfer of this proton to the hydrazine molecule as the reduction takes place. The results show that coordination of a carboxylate ligand to the Mo atom in the model cubanes does not "block" reactivity and suggests that the homocitrate ligand bound to the Mo atom in the nitrogenase cofactor may play a similar, proton-transfer, role. In addition to the catalytic reduction of hydrazines by the single cubane catalysts, a catalytic disproportionation reaction also takes place where both electrons and protons are supplied by hydrazine, (Table 2).

Table 2. Catalytic disproportionation of N_2H_4 in CH_3CN by $[(Cl_4\text{-cat})MoFe_3S_4Cl_3(CH_3CN)]^{2-}$ at ambient tempeature.

N_2H_4/catalyst[a]	time (min)	NH_3[b]	% reduction[c]
10	30	6.2	46
20	30	7.1	27
40	30	11.0	21

a) Cubane concentration $2x10^{-4}$M. b) Ammonia yields reported as equivalents of catalyst and were quantified by the indophenol method. c) Maximum yield is 4/3 NH_3 per N_2H_4 from the reaction $3N_2H_4 \Rightarrow NH_3 + N_2$.

The disproportionation of N_2H_4 is not observed when, in the $[(Cl_4\text{-cat})MoFe_3S_4\ Cl_3(L)]^{n-}$ cubane catalyst, substitution-inert ligands (L = CN^-, PEt_3 etc) are coordinated to the Mo atom. In contrast, the catalytic reduction by the same cubanes is not affected by the presence of Mo-bound substitutionally inert ligands. The results show (Table 3) that in the disproportionation reaction, where no external source of protons is added, the Mo atom is effectively blocked and the coordination/reduction of N_2H_4 does not take place to a significant extent. The catalytic reduction of N_2H_4 with the same clusters is possible, however, because now the externally added protons add to the O atoms of the Mo-bound Cl_4-catecholate ligand and in the process generate a coordination site on the Mo atom thus allowing the N_2H_4 molecule to coordinate and undergo reduction.

Table 3. Catalysis of hydrazine disproportionation and reduction reactions with the $[(Cl_4\text{-cat})MoFe_3S_4\ Cl_3(L)]^{n-}$ cubanes

Catalyst (L)[a]	N_2H_4/catalyst	Reduction [b,c]	Disporportionation[b,c]
CH_3CN	10	14.6 (73%)	8.6 (64%)
CN^-	10	8.1 (40%)	1.1 (8%)
CH_3NH_2	10	8.6 (43%)	1.2 (9%)
Cl^-	10	7.2 (36%)	2.4 (18%)
PEt_3	10	8.0 (40%)	0.7 (5%)

a) Concentration of catalyst was $2x10^{-4}$M. b) All reactions were analyzed for NH_3 after 60 min. c) Ammonia quantified by the indophenol method.

The site of N_2H_4 reduction also is the site of H^+ reduction. Indeed N_2H_4 is an inhibitor of H^+ reduction (Table 4). This inhibition is less pronounced with substituted hydrazines that are bound to the Mo atom via the R substituted N atom. Steric interactions of the R group with the catecholate ligand promote dissociation of the $RNHNH_2$ ligand and allow for the reduction of H^+ that most likely hydrogen-bonds to the Mo-bound O atoms of the catecholate ligand.

Table 4. Proton reduction/hydrogen evolution catalyzed by $[(Cl_4\text{-cat})MoFe_3S_4Cl_3(CH_3CN)]^{2-}$ (I) in CH_3CN in the presence of $Co(Cp)_2$ and Lut.HCl as sources of e^- and H^+, respectively.

Catalyst	NH_2NHR	$RNHNH_2$/ catalyst	NH_3[a]	H_2[b]
None	None	0	0	1.0 (5%)
(I)	None	0	0	20.0 (100%)
(I)	R=H	10	19 (95%)	1.0 (5%)
(I)	$R=CH_3$	10	10 (50%)	10.0 (50%)

a) Yields reported as equivalents of ammonia per equivalent of (I). b) Hydrogen yields are reported as equivalents per equivalent of (I) and were quantified by gas chromatography.

The VFe_3S_4 cubanes also are effective in the catalytic reduction of hydrazine to ammonia (Malinak et al., 1995). Catalytic reductions with $[(DMF)_n(L)_{(3-n)}VFe_3S_4Cl_3]^{2-}$ (n=3; n=2, $L=PEt_3$; n=1, L_2=bipy; n=0, L_3=tris-pyrazolylborate) show the rate of the reaction increasing with the number of substitutionally labile DMF molecules coordinated to the V atom (Table 5).

Table 5. Catalytic reduction of hydrazine by the $[(DMF)_n(L)_{(3-n)}VFe_3S_4Cl_3]^{2-}$ clusters in CH_3CN in the presence of $Co(Cp)_2$ and Lut. HCl as sources of e^- and H^+, respectively.

N_2H_4/catalyst;L,n	time(min)	NH_3[a,b]	% reduction[c]
10; n=3	120	20.0	100
10; $L=PEt_3$, n=2	120	9.4	47
10; L_2=bipy, n=1	120	3.4	17
10; L_3=tris-pyrazolylborate, n=0	120	0.0	0
10[d]; n=3	120	2.4	24

a) Concentration of catalyst was $1x10^{-4}$ M. b) Ammonia reported as equivalents produced per equivalent of catalyst, quantified by the indophenol method. d) Reduction of phenylhydrazine.

The data again indicate that the catalysis involves the V atom since the reduction potentials for the $[(DMF)_n(L)_{(3-n)}VFe_3S_4Cl_3]^{2-}$ clusters (CH_3CN solution, Pt electrode vs SCE) are nearly the same at -1.2V. The $[(DMF)_3VFe_3S_4X_3]^{2-}$ cubanes (X =Cl, Br, I) catalyze the reduction of hydrazine to ammonia with a rate that does not change with the Fe-bound halide terminal ligands.

Catalytic reduction of acetylene to ethylene.

The $MoFe_3S_4$ single cubane SC has been found effective in the catalytic reduction of acetylene to ethylene and a small amount of ethane by a process that follows saturation kinetics with Km=17.9 mM and Vmax = 1.1×10^{-4} M/min. A kinetic study as a function of temperature was carried out and activation parameters, ΔG, ΔH and ΔS were determined as 19(1) Kcal/mole, 9(1)Kcal/mole and -32 e.u., respectively (Laughlin, Coucouvanis, 1995). As is the case for the reduction of acetylene by nitrogenase, the acetylene reduction by the model cubane gives cis-ethylene as verified by isotopic substitution experiments. The acetylene reduction slows down upon addition of Et_3P, CN^- and CO (ligands that bind preferentially to the Mo atom) but is not completely turned off. In fact, the rate of acetylene reduction with the Mo site blocked is the same as the rate of reduction found when the $[Fe_4S_4Cl_4]^{2-}$ cubane is used as a catalyst. The data suggest that the reduction of acetylene is occuring at both the Mo and Fe sites, albeit at different rates.

Stoichiometric reduction of the bridging CN^- ligand in the doubly-bridged double cubanes to methane and ammonia.

The $[(Cl_4\text{-cat})MoFe_3S_4Cl_3(CN)]^{3-}$ and $[(Cl_4\text{-cat})MoFe_3S_4Cl_3]_2(\mu\text{-}CN)]^{5-}$ clusters do not react with $Co(Cp)_2$ in the presence of Lut.HCl. The $\{[(Cl_4\text{-cat})MoFe_3S_4Cl_2]_2(\mu\text{-}CN)(\mu\text{-}S)]\}^{5-}$doubly-bridged double cubane, DBDC, however undergoes reduction to give methane and ammonia (Coucouvanis et al., 1995). This reduction is preceded by protonation of the cluster. The protonated $\{[(Cl_4\text{-cat})MoFe_3S_4Cl_2]_2(\mu\text{-}CN)(\mu\text{-}S)]H\}^{4-}$ cluster has been isolated and spectroscopically characterized. The bimetallic activation and reduction of the CN triple bond in the DBDC represents the first example of a six electron reduction in a Fe/Mo/S cluster.

Summary and Conclusions

Our studies with model Fe/M/S complexes thus far have demonstrated that: a) the heterometal (Mo, V) is very important in hydrazine reduction and the Fe atoms are not involved in the reduction; b) the Mo-bound tridentate citrate ligand does not preclude reactivity at the Mo atom, on the contrary, it enhances the rate of substrate reduction possibly by participation in intramolecular proton transfer to the reduced substrate; c) the hydrazine molecule in a bridging mode is unreactive to reduction, presumably due to the unavailability of lone pairs of electrons that are needed for an essential protonation step; d) different substrates may interact with and undergo reduction at different sites within the MFe_3S_4 cubanes; and e) multiply bonded substrates may necesitate bimetallic activation. The extent to which these findings are relevant to the function of the nitrogenase cofactor remains to be established. However, the basic chemical principles that underlie the reactivity of the model clusters also apply to the structurally similar nitrogenase cofactor.

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INCORPORATION OF THE IRON AND SULFUR FROM NIFB-CO INTO THE IRON-MOLIBDENUM COFACTOR OF DINITROGENASE

ALLEN, R.M., CHATTERJEE, R., LUDDEN, P.W. AND SHAH, V.K.
Department of Biochemistry and Center for the Study of
Nitrogen Fixation, College of Agricultural and Life
Sciences, University of Wisconsin-Madison, Madison, WI, 53706, USA

NifB-co, the product of the *nifB* gene, is involved in the biosynthesis of the iron-molibdenum cofactor (FeMo-co) of dinitrogenase. Incorporation of ^{55}Fe and ^{35}S from NifB-co into FeMo-co was investigated using the *in vitro* FeMo-co synthesis system. Fe and S from NifB-co were incorporated into dinitrogenase only under conditions where all components required for FeMo-co synthesis were present. Incorporation of Fe and S from NifB-co into dinitrogenase was not observed in control experiments in which apodinitrogenase was initially activated with purified, unlabelled FeMo-co or in assays where NifB-co was oxygen-inactivated prior to addition to the synthesis system. These data clearly demonstrate that Fe and S from active NifB-co are specifically incorporated into FeMo-co of dinitrogenase and provide the first biochemical identification of an FeS donor for FeMo-co synthesis.

Under different *in vitro* FeMo-co synthesis conditions, Fe and S from NifB-co were associated with at least two other proteins that are involved in the formation of active dinitrogenase (NIFNE and γ protein). These results suport a FeMo-co biosynthetic model in which FeMo-co is assembled on NIFNE prior to being transferred to the γ protein, which shuttles the FeMo-co to apodinitrogenase.

REDOX GATING OF ELECTRON TRANSFER IN THE NITROGENASE VFE-PROTEIN FROM *AZOTOBACTER VINELANDII*

BLANCHARD, C. Z., TITTSWORTH, R. C., AND HALES, B. J.
Department of Chemistry, Louisiana State University,
Baton Rouge, LA 70803-1804 USA

Recently our laboratory demonstrated that *A. vinelandii* synthesizes two different forms of V-nitrogenase component 1, termed Av1'$_A$ and Av1'$_B$. Densitometer traces of SDS gels revealed a difference in the composition of the major α and β polypeptides of the two forms, with Av1'$_A$ having a composition $\alpha\beta_2$ while composition of Av1'$_B$ was $\alpha_2\beta_2$, similar to that of component 1 from Mo-nitrogenase. Metal analysis further showed that Av1'$_A$ possessed only one cofactor (FeVco) and one P-cluster compared to the two cofactors and P-clusters of Av1'$_B$. In spite of these differences, both protein forms showed the same substrate reduction patterns for H^+, N_2, C_2H_2 as well as CO inhibition.

Oxidation of Av1'$_B$ with thionine resulted in a stepwise oxidation of the P-clusters and then the cofactors, similar to the oxidation of the MoFe-protein (Tittsworth, Hales, 1993). However, with Av1'$_A$, a unique redox-induced structural change (as monitored by EPR spectroscopy) occurred which reversed the flow of electrons between the cofactor and P-cluster. Stepwise addition of thionine initially oxidized the cofactor while leaving the P-cluster reduced. After the cofactors had been oxidized by one equivalent each, the next addition of thionine caused the cofactors to be reduced back to their original native state with the simultaneous oxidation of the P-clusters. These results can be explained as follows. Form Av1'$_A$ exists as two different structures, called Av1'$_{A1}$ and Av1'$_{A2}$. Initially the protein is in structure Av1'$_{A1}$. In this structure the cofactor oxidizes prior to the P-cluster (while in form Av1'$_{A2}$ the reverse is true where the P-clusters oxidize before the cofactor). Following oxidation of the cofactors in Av1'$_{A1}$, the next addition of oxidant establishes the equilibrium P ¤ $P^+ + e^-$. The formation of P^+ induces Av1'$_{A1}$ to change to Av1'$_{A2}$ resulting in an electron flow from the P-cluster into the previously oxidized cofactor.

We have termed this phenomenon "redox gating of electron flow". This effect suggests that an oxidation change in a metal cluster can initiate a structural change in a protein. In the above situation the structural change has driven an electron into the cofactor from the P-cluster, a reduction step that may be important in the mechanism of nitrogen fixation.

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THE P CLUSTERS OF *A. VINELANDII* MOLYBDENUM NITROGENASE

CANTWELL, J.S. (1), FISHER, K. (1), DEAN, D.R. (1),
FINNEGAN, M. (2), JOHNSON, M.K. (2) AND NEWTON, W.E. (1)
(1) Department of Biochemistry and Anaerobic Microbiology, Virginia Tech, Blackburg, VA 24061-0308, USA
(2) Department of Chemistry, University of Georgia, Athens, GA 30602, USA

Prior to the recent solution of the crystal structure of the *A. vinelandii* MoFe protein, potential P cluster and FeMoco ligands had been identified by metallocluster extrusion experiments and primary sequence comparisons. Preliminary site-directed mutagenesis studies showed that substitution of some of the proposed P cluster ligands resulted in altered proteins that remained capable of diazotrophic growth, albeit at a reduced rate.

We report pre-steady-state, steady-state and spectroscopic data from purified proteins containing substitutions at the α88Cys, β153Cys and β188Ser positions in attempts to determine the role of P clusters in nitrogenase catalysis. All altered MoFe proteins show kinetic parameters from steady-state and pre-steady-state assays that are consistent with perturbed inter- and intra-molecular electron-transfer pathways and a tighter Fe protein-MoFe protein complex. MCD indicates that no substitution affects the FeMoco site, but that each substitution affects the P cluster's electronic and magnetic properties differently. The α88C ⇨ G MoFe protein in the dithionite reduced state has its P cluster(s) in a 1-electron-oxidized state, suggesting significant perturbation of either the redox properties or composition (or both) of the P clusters.

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ALTERNATIVE PHOTOCHEMICAL SYSTEMS FOR NITROGEN AND ACETYLENE REDUCTION CATALYZED BY NITROGENASE.

DRUZHININ S.Yu., SYRTSOVA L.A., UZENSKAYA A.M.,
NADTOCHENKO V.A., LIKHTENSTEIN G.I.
Institute of Chemical Physics of Russian Academy of Sciences,
Chernogolovka, Moscow region, 142432, Russia.

In order to carry on further investigations of elementary stages of nitrogen fixation more than 10 photochemical systems have been invented and tested in the relation to their capacity to support the reduction of nitrogenase substrates. Each system contained a unique mixture of an electron donor (NADH, EDTA or glutathione with ascorbate) and a photosensitizer (eosin, erythrosin, fluorescein, Zn-porphyrin or tetrasulfotetraphenilporphyne). It has been found that the systems eosin-NADH, erithrosin-NADH and Zn-porphyrin - NADH provided necessary conditions for nitrogenase action in the presence of MgATP. The study of the eosin(0.04 mM) - NADH(0.05 mM) system by the method of impulse laser photolysis showed that there occured a formation of anion-radicals of eosin ($k = 3x10^{-4}\ s^{-1}$) in the presence of nitrogenase protein components. During the enzyme reaction anion-radical forms (with $E_{1/2}$ = - 0.58 V vs. NHE) reduced the iron-protein (with $E_{1/2}$ = - 0.42V vs. NHE) and then nitrogenase passed electrons to nitrogen or acetylene or other substrates. The formation of ammonia from nitrogen and ethylene from acetylene has been established. The rate of photoreduction (per electron) of acetylene or nitrogen under identical conditions was shown to be similar. At the nitrogenase components ratio equal to 1 the rate of photochemical reduction is higher than in the presence of dithionite: the total electron flux through nitrogenase being increased about 2-fold. Thus, it has been done a suggestion that in this case the nitrogenase complex (1:1)works without dissociation.

COVALENT MODIFICATION OF NITROGENASE MoFe PROTEIN BY ADP.

EADY, R.R., MILLER, R.W., GORMAL, C.,
FAIRHURST, S. AND SMITH, B.E.
The Nitrogen Fixation Laboratory, The University of Sussex
Brighton BN1 9RQ U.K.

The role of MgATP in nitrogenase function is not clearly established. It is required for electron transfer from the Fe protein to MoFe protein, and for substrate reduction, reactions which are potently inhibited by MgADP. The binding of MgATP or MgADP to Fe protein produces a conformational change and a ~ 100 mV decrease in the mid-point potential of the 4Fe4S cluster. The x-ray crystal structure shows the nucleotide-binding site to be at the subunit interface some 20 Å away from the redox centre (Georgiadis et al.,1992). The hydrolysis of MgATP requires the presence of both Fe and MoFe proteins.

We have previously reported binding of MgATP and MgADP to MoFe proteins demonstrated by gel-filtration binding studies, and also ^{32}Pi exchange reactions from orthophosphate into both ATP and ADP ((Miller et al.1980; Miller, Eady, 1989). The interaction with MgADP is complex, with up to 4 ADP molecules being bound, and 2 of these remaining tightly associated with the protein on gel filtration (Miller et al., 1993). Here we report NMR data on the origin of the non-dissociable binding of MgADP to MoFe proteins and evidence for a possible involvement of lysine close to the MgADP-binding site.

MgADP reacts slowly with the MoFe protein of *Klebsiella pneumoniae* (Kp1) over 2-3 h to produce a stable conjugate and PO_4^{2-}. The reaction was monitored by the accumulation of 8-^{14}C adenine ring compound and by pulsed ^{31}P NMR. The conjugate was characterized by a resonance at - 1.1 ppm and was assigned to AMP covalently bound to Kp1, which retained full activity. The temperature dependence of the broadening of the ^{31}P NMR spectra is consistent with a mobile surface location of the AMP on Kp1. The Kp1-AMP species is unstable, and over 6 h degrades to form AMP and Kp1. MgADP, but not MgATP, protected Kp1 from inactivation by the lysine-modifying reagent *o*-pthalaldehyde. These data clearly indicate that the interaction of nucleotides with nitrogenase components is more complex than is often considered. The significance of the Kp1-AMP species to nitrogenase catalyzed reactions remains an open question.

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SUBSTITUTION OF β-TYR^{98} OF THE *A. VINELANDII* NITROGENASE MoFe PROTEIN BY β-HIS^{98} ALTERS INTRAMOLECULAR ELECTRON TRANSFER

FISHER, K., NEWTON, W.E., DEAN, D.R. AND PETERS, J.W.
Department of Biochemistry and Anaerobic Microbiology,
Virginia Tech, Blackburg, VA 24061-0308, USA

The recent X-ray crystal structure of the *A. vinelandii* MoFe protein has given us the opportunity to examine possible pathways of electron transfer between the P-clusters and FeMoco. Since little is known about intramolecular electron transfer, we have identified a tyrosine residue that is in direct line with the shortest apparent pathway between the two metal centers. An aromatic residue was chosen because it has been suggested that aromatic residues may facilitate electron transfer in proteins. Several amino acid substitutions (Phe, His and Leu) were made for this tyrosine residue in the β-subunit. Steady state data show that substituting tyrosine by another aromatic or an aliphatic residue causes no significant affect on enzyme catalysis, whilst changing this tyrosine to a histidine disrupts intramolecular electron transfer. Pre-steady state stopped-flow kinetic data obtained with this tyr ⇨ his altered protein shows that perturbation of intramolecular electron flow occurs following primary electron transfer.

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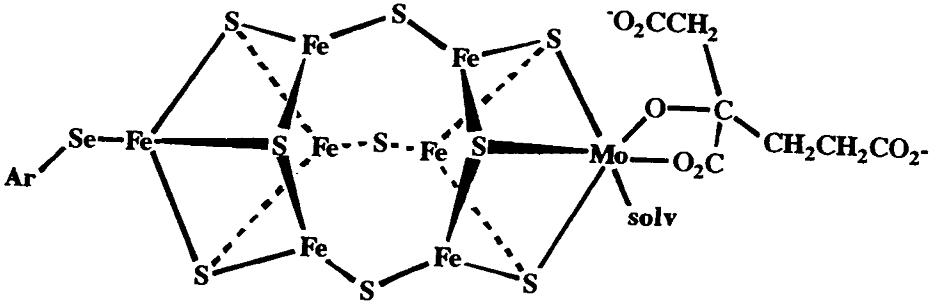
THIO- AND SELENO-PHENOL BIND TO FE OF FeMoco OF NITROGENASE FROM *KLEBSIELLA PNEUMONIAE*

GORMAL, C.A.[a] , HARVEY, I.A.[b] , HASNAIN, S.S.[b] , RICHARDS, R.L.[a] , SCHNEIDER, R.[a], SMITH, B.E. [a], AND STRANGE, R.W. [b]
[a] JIC Nitrogen Fixation Laboratory, University of Sussex, BN1 9RQ, UK
[b] Synchrotron Radiation Research Division, CCL Daresbury Laboratory, Warrington WA4 4AD, UK

We and others (Conradson et al., 1994) have been studyng the reactions of the isolated metal cluster from the active site of molybdenum nitrogenase (FeMoco), particularly with thiols and selenols. For FeMoco from *Klebsiella pneumoniae*, our EPR and Fe- and Se-K edge XAFS data, at an FeMoco: ArEH ratio of 1:1, are consistent with one ArE ligand (Ar=aromatic group, E=S or Se) binding to an iron of FeMoco and not to Mo. For PhSe, d(Fe-Se) = 2.34 A and d(Se-C) = 1.90A. Whilst which of the seven irons in FeMoco (Chan et al., 1992) binds to ArE is uncertain, the most likely site is as shown in the Figure. As observed for FeMoco from *Azotobacter vinelandii* (Conradson et al., 1994), we find the Se-K edge XAFS data at a PhSeH:FeMoco ratio of 10:1 are difficult to interpret. The greater amplitude in the region where Fe absorbs might indicate weak binding of PhSe at a face of the FeMoco cluster.



solv = solvent such as N-methylformamide (NMF)

Reaction of FeMoco with the dithiols $C_6H_4(SH)_2$ -1,2 and $MeC_6H_3(SH)_2$-3,4 precipitates some S_8 and gives new red compounds with no FeMoco activity. NMR, EPR and UV/vis spectroscopic data and elemental analysis indicate NEt_4 salts which contain dithiolate and NMF and are structurally related to FeMoco. The Fe-to-Mo ratio in these products is close to 6:1. On exposure to air, the red products change to blue materials with a lower Fe:Mo ratio.

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STRUCTURAL REQUIREMENT FOR THE CLUSTERS TO BE RECONSTITUTED WITH FeMoco–DEFECIENT MOLYBDENUM –IRON PROTEIN

HUANG J. F., LUO A. L. HU C. Z., WANG Z. P., AND LI J. G.
Institute of Botany, Academia Sinica, Beijing, 100044, China
XU J. Q., NAN Y. M., LIU Y. and CHEN Y. D.
University of Jilin., Changchun, 130021, China

By incubating the reduced MoFe protein from *A. vinelandii* with o–phenanthroline under air and chromatographying the incubated solution on Sephadex G–25 column, inactive MoFe protein MoFe(C,O) could be obtained (Huang et al.,1991). The inactivation of the treated protein mainly resulted from a partial deficiency in metalloclusters and an oxidative damage of -SH groups. However, its C_2H_2 reduction activity was remarkably recovered by the incubation not only with the reconstituent solution containing Mn (Huang et al., 1994), but also with a mixture of 4Fe: 4S clusters (Liu,1992) and another cluster which had two structure units-1Mo: 3Fe: 4S–bridged by three $-OCH_3-$ at the Mo atoms (Nan, 1992). But neither the reconstituent solution nor the mixture could reactivate apo–MoFe proteins from the mutants deleting nifE and nifH genes (Robinson et al.,1987; Brigle et al.,1987) and from the mutant UW45, which could be reactivated by the FeMoco extracted from the MoFe protein (Shah and Brill, 1977). The results indicated that the FeMoco deficient MoFe protein from these mutants seemed to be reconstituted only by the clusters which were probably similar in structure to the FeMoco;and the partially metalloclusters–deficient MoFe protein could be reconstituted by the clusters with a certain kind of stucture and composition because the apo–MoFe protein might have a suitable structure and flexibility for “Assembly Parts” formed in the reconstituent solution to be changed into FeM–cofactor.

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A NEW NITROGEN FIXATION SYSTEM: PROBABLY IN VITRO ASSEMBLING FeMco BY REPLACING Mn AND Cr FOR Mo IN NITROGENASE

HUANG J.F., WANG Z.P., LUO A.L., ZHONG Z.P., and LI J.G.
Institute of Botany, Academia Sinica, Beijing, 100044, China
DONG M. L., DU H. S., LI J. H. and DU S. B.
Institute of Atomic Energy, Beijing, 102413, China
YU L.J., ZHANG F. Z., XU L. S. and CAI Q. R.
University of Xiamen, Xiamen, 361005, China

By incubating the reduced MoFe protein from *A. vinelandii* with o-phenanthroline under air, the inactive protein–MoFe(C,O) could be obtained. The percentage of the activity loss was about 10% higher than that of Fe loss for the MoFe(C,O)and DTT could keep the protein increasing 10–20% activity. It was indicated that some of metalloclusters and –SH groups in the protein had been damaged. After Incubating the MoFe(C,O) with a reconstituent solution composed of $^{99}MoO_4^{2-}$, ferric homocitrate, Na_2S and DTT, and chromatographying on Sephadex G–25 and DEAE columns, the reconstituted protein could be obtained. The measurement of ^{99}Mo in the protein using Perturbed Angular Correction showed that the quadrupole coupling constant (412 MHz) and the asymmetry parameter (0.49) of 41% out of ^{99}Mo atoms were similar to those obtained from the intact cells of *K. pneumoniae* (Mottner et al., 1993). When incubating the MoFe (C,O) with other reconstituent solutions in which MnO_4^{1-}, CrO_4^{2-} and MoO_4^{2-} were substituted for $^{99}MoO_4^{2-}$, the coresponding reconstituted proteins could be also obtained. Their C_2H_2, H^+-reduction activity, and N_2 reduction activity which were examined by the difference of H_2 evolution under Ar and N_2 could be well recovered, and their native anaerobic PAGE behavior were also restored, and their CD or EPR spectra could be significantly restored or changed but were somewhat different from that of the reduced MoFe protein. However, neither the activity nor spectra of the protein treated with the solution in which WO_4^{2-} substituted for $^{99}MoO_4^{2-}$ were remarkably recovered. The results indicated that the reconstituted protein solutions, of which the activity had been restored, probably contain new protein in which Mn, Cr and $^{99}MoO_4^{2-}$ substituted for Mo. Therefore, it seems to be reasonable to suggest that (1) Mo in FeMoco could be substituted by Mn or Cr *in vitro*; (2) the assembled MnFe or CrFe protein might be similar in function but different in structure from the MoFe protein.

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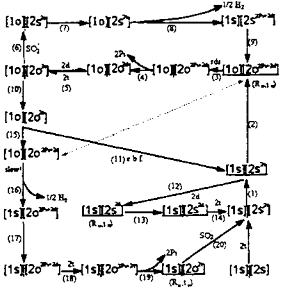
ELECTRON AND PROTON TRANSPORT IN NITROGENASE CATALYSIS⁺

HUANG J.W. ZHANG H.T. WAN H.L.* TSAI K.R.*
Department of chemistry, Xiamen University, Xiamen 361005, China.
⁺Supported by a State Key Project on Symbiotic Nitrogen Fixation.
*Authors for correspondence.

Under steady-state reduction of nitrogenase substrates (proton, or N_2, or HC≡CH) with sufficient supply of reductant (e.g., dithionite) and MgATP(denoted as **t**), the redox states of MoFe-protein [**1**] and Fe-protein [**2**] (*Kp1/Kp2*≈1/1) are 60-90% in the epr-silent, fully-reduced state [**1**o] and ≤ 60% in the epr-silent, reduced state [**2**o], respectively; while with deficient supply of reductant, or ATP, the system will end up, respectively, with [**1**s] and [**2**o] states, or [**1**s] and [**2**s] states [Smith et al., 1973]; and in the steady-state reduction of the substrate N_2O (normally an easily reducible molecule), the redox state of the enzyme components also appear to be the same as with N_2 (normally a very inert molecule). So it is very likely that these redox-state changes will follow the same patterns for all other substrates, regardless of the difference in reducibility of these substrates. It is known that complexation of [**1**] with 2**t**-bound [**2**] will produce appropriate conformational change in [**1**], leading to ATP hydrolysis followed immediately by transport of an e^- from [**2**s] to [**1**s], or ultimately to substrate molecule bound to [**1**]. In *in vitro* biosynthesis or insertion of FeMo-co, ATP and [**2**] are both required, but normal ATP hydrolysis and electron transfer from [**2**] to [**1**] are not requisite [Gavini and Burgess, 1992]. In the functioning nitrogenase system with large excess of [**1**], most of the [**1**] still appear to be in the fully reduced state, [**1**o], but substrate reduction efficiency is very low and ATP hydrolysis excessive.

Electron back-flow from [**1**o] to [2**t**-**2**o] has been suggested to account for ATP/2e >> 4 [Tsai, 1976]. It may be postulated that the conformational change resulting from association of [**1**] with [2**t**-**2**] is required for completing the coupled-electron-and-proton transport pathway from [**2**] to [**1**] in case of substrate reduction (which requires the transport of an electron and a proton in each cycle), or the other way around in case of electron back-flow, involving P-cluster pair and M-cluster in either case. A mechanism of ATP-driven coupled-electron-and-proton transport is proposed as shown in Figure.



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PROBABLE SITES OF DINITROGEN AND ACETYLENE BONDING TO NITROGENASE[+]

HUANG J.-W.[1], ZHANG F.-Z.[2], ZHANG H.-T.[1], XU L.-S.[2]
LIU A.-M.[1], WAN H.-L.[*1], TSAI K.R.[*1]
Department of Chemistry[1], Department of Biology[2], Xiamen University,
Xiamen 361005, China. [+]Supported by State Key Project on ONMSNF

Based on the K–R model [Kim & Rees, 1992] & the principles of coordination & catalysis, it has been proposed [Tsai & Wan, 1995] that FeMo-co (M-cluster) is a labile-mouthed ("Y" may be μ-SH) cluster cage with molecular-sieve shape-selective-binding of substrates (inside the cage: [6Fe]-site for HC≡CH, cyclopropene, & diazirine; [Mo-3Fe]-site for N≡N only; outside the cage: [2Fe]-site left by "Y" for MeC≡CH etc.). In this work, the effect of inhibition by N≡N on the cis selectivity in *Av*-catalyzed reductive dideuteration of HC≡CH in D_2O is studied.

The non-inhibition by ethene was confirmed, indicating that N≡N is coordinated inside the cage, not at the [2Fe]-site. Strong inhibition by N≡N of HC≡CH reduction was also confirmed, in accord with relative binding strengths (~$1/K_M$) and the proposal that both substrates are essentially coordinated on active sites inside the cage, with the [Mo-3Fe]-site available only to strong coordination and reduction of N≡N.

Most significantly, the observed decrease in cis-selectivity in nitrogenase catalyzed reduction of HC≡CH in D_2O with decreasing pC_2H_2 in Ar, and much bigger decrease in cis-selectivity with decreasing pC_2H_2 in N≡N, as shown by the relative FTIR peak-areas of the cis- & trans-HDC=CDH at 843 & 988 wave numbers, respectively [Hardy et al., 1968], the observed trans/cis ratios being: 0.0069, 0.016, & 0.019 at pC_2H_2 **(in Ar)** = 1/14, 1/109, & 1/218 atm., respectively; and 0.032 & 0.050 at pC_2H_2 **(in N≡N)** = 1/109 atm (81% inhibition) & 1/218 atm (89% inhibition), respectively. Based on known binding strengths & cis reduction selectivities of HC≡CH (>99 %) and MeC≡CH (~28%), ~1% of total HC≡CH reduction might take place at the [2Fe]-site with roughly 50%-50% cis & trans products, and practically not inhibited by N≡N coordination, nor by H_2 sweeping, the expected trans/cis ratios would be about 0.005 for about 10% acetylene in argon, and about 0.027 & 0.048, for pC_2H_2 **(in N≡N)**=1/109 & 1/218 atm, respectively, The results provide a very strong support for the proposed binding sites.

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MOLECULAR STUDY OF A PUTATIVE MEMBRANE COMPLEX INVOLVED IN ELECTRON TRANSFER TO NITROGENASE IN *RHODOBACTER CAPSULATUS*

HUGO, N., JOUANNEAU, Y., WILLISON, J.C. AND VIGNAIS, P.M.
Laboratoire de Biochimie Microbienne, CEA and CNRS URA 1130,
C.E.N.G., 17 rue des Martyrs, F-38054, Grenoble cedex 9, France.

In most N_2-fixing bacteria, including *Rhodobacter capsulatus*, the electron transfer pathway to nitrogenase is poorly understood. A genetic analysis revealed the presence of six genes, called *rnfA* to *rnfF*, coding for the components of a putative *nif*-specific electron transport system in *R. capsulatus* (Schmehl et al., 1993). The *rnf* gene products are predicted to be transmembrane polypeptides (RnfA, RnfD, RnfE and RnfF) or proteins containing iron-sulfur clusters (RnfB and RnfC), which may assemble to form a membrane complex.

Besides, a ferredoxin called FdI, has been identified as the most likely electron donor to nitrogenase (Jouanneau Y., Meyer C., Naud I. and Klipp W, unpublished results). The characterization of the *rnf* gene products has been undertaken. Each of them has been individually cloned into the *E.coli* expression vector, pET15b, which allows the production of recombinant proteins bearing a poly-histidine tag fused to the N-terminus. The RnfB protein could be overproduced under standard conditions of induction. It was synthesized as an insoluble polypeptide and could be purified under denaturing conditions using ion-affinity chromatography. Thrombin digest yielded a smaller polypeptide exhibiting the expected N-terminal sequence of RnfB. The recombinant RnfB product contained no iron-sulfur cluster, despite the presence of appropriate cysteine ligands in the sequence. Overexpression of *rnfC* yielded two recombinant polypeptides by SDS-PAGE, one of which having the expected size (55 kDa), the other being much smaller (25 kDa). The latter polypeptide was more abundant and represented a truncated N-terminal part of RnfC. The *rnfA* gene expression gave rise to a 14 kDa band which was detectable on SDS-PAGE only upon radioactive labeling. Since that polypeptide had a size smaller than RnfA (20.4 kDa), it likely resulted from a proteolytic degradation of the recombinant product.

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ELECTRON TRANSPORT TO NITROGENASE : STUDIES ON SOME OF THE PROTEINS INVOLVED IN *R. CAPSULATUS*

JOUANNEAU, Y., HUGO, N., ARMENGAUD, J., NAUD, I., MEYER, C.
AND WILLISON, J.
Laboratoire de Biochimie Microbienne, CEA and CNRS URA 1130,
C.E.N.G., 17 rue des Martyrs, F-38054 Grenoble cedex 9, France.

In most nitrogen fixing bacteria including phototrophs, the electron transport system providing reductants to nitrogenase is poorly understood. Our studies aim to identify the proteins responsible for this electron transport in *Rhodobacter capsulatus*. As many as six distinct ferredoxins have been isolated and characterized from this bacterium, and the genes encoding these proteins have been cloned (Grabau et al., 1991; Jouanneau et al., 1993; Armengaud et al., 1994; Naud et al., 1994). Genetic and biochemical analyses indicated that one ferredoxin, called FdI, serves as electron donor to nitrogenase. The interaction between FdI and dinitrogenase reductase (Rc2), has been investigated through cross-linking experiments and *in vitro* electron transfer assays. To identify residues of FdI that participate in the interaction with Rc2, site-directed FdI mutants were generated. While the mutants obtained showed minor changes of the redox and spectroscopic properties, replacements of some charged residues, predicted to be at the surface of the molecule, resulted in variants with impaired interaction or activity towards Rc2.

The biochemical pathway allowing the regeneration of reduced ferredoxin (FdI) in *R. capsulatus* has also been investigated. Our working model postulates the existence of a *nif*-specific membrane-bound complex which would serve as a key intermediate coupling substrate oxidation to reduction of the soluble ferredoxin. The components of this putative membrane complex are encoded by a set of at least six genes called *rnfA* to *rnfF* which belong to two contiguous operons (Schmehl et al., 1993). These genes have been individually cloned into T7-based expression vectors in order to induce the overprodution of the corresponding polypeptides in *E. coli*. Such a strategy allowed to isolate two of the proteins of interest, encoded by *rnfB* and ORF14, respectively. The *rnfB* product was purified from inclusion bodies as a 25 kDa polypeptide devoid of Fe-S cluster, despite the presence of cysteines forming two potential binding sites for such clusters. The ORF14 product was isolated as a 50 kDa polypeptide containing one FMN group per subunit. This flavoprotein was reducible by dithionite or 5-deazaflavin, but not by NAD(P)H.

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USE OF FLUORESCENT ANALOGUES TO INVESTIGATE CONFORMATIONAL CHANGES IN THE FE-PROTEIN OF *KLEBSIELLA PNEUMONIAE* NITROGENASE

HAYLEY C. KNIGHTS AND ROGER N. F. THORNELEY
BBSRC Nitrogen Fixation Laboratory, University of Sussex, Falmer, Brighton
BN1 9RQ, U.K.

Work on p21^{ras} using the fluorescent analogue 2'(3')-*O*-(*N*-methylanthraniloyl)GTP (mantGTP) has provided real time measurements of the binding of mantGTP to p21^{ras} (observed as a 3.2-fold increase in fluorescence) and a subsequent isomerisation step (seen as a 10% loss of fluorescence) which was not due to the hydrolysis of the analogue (Neal *et al.*, 1990). The fluorescent analogue mantADP has been used to investigate conformational changes in the Fe-protein of *K. pneumoniae* nitrogenase induced by nucleotide binding.

On mixing a two-fold excess of mantADP with oxidised Fe-protein in the stopped-flow spectrophotometer a loss of 15% fluorescence is observed within the mixing time (3 ms). This is followed by an exponential loss of fluorescence (50%) with k_{obs} increasing from 20 to 50 s^{-1} over a 18-fold increase in mantADP concentration. This change is most likely associated with a conformational change in the protein rather than the nucleotide binding step itself. A large excess of ADP displaces bound mantADP from the oxidised Fe-protein such that the fluorescence of the system is recovered by >94%. The fluorescent analogue mantADP consists of a 35% : 65% mixture of 2'- and 3'- isomers (Cremo *et al.*, 1990) which, when free in solution isomerise at an observed rate of less than $1s^{-1}$ at pH 7.0 and 22°C (Eccleston *et al.*, 1990). A migration of the mant fluorophore from the 3'- to the 2'- position (the former isomer is twice as fluorescent) when bound to the oxidised Fe-protein, could account for the initial rapid loss of fluorescence. However this would require the rate of acyl migration to become *ca.* 250 times faster on the protein than in solution.

To avoid problems of kinetic assignment when two isomers are present, the fluorescent analogue 3'-mantdeoxyADP (mantdADP) has been used under the same conditions. On mixing a two-fold excess of mantdADP with oxidised Fe-protein no loss of fluorescence occurs within the mixing time. A biphasic loss of fluorescence (35% in total) was observed with k_{obs} increasing from 40 to 85 s^{-1} for the first exponential and from 1 to 5 s^{-1} for the second over a 18-fold increase of mantdADP. Since only a *ca.* 2-fold increase in k_{obs} occurred for an 18-fold increase in either mantADP or mantdADP concentration, these fluorescence changes are probably not associated with the binding of the nucleotides, but are associated with a conformational change in the protein (Ashby and Thorneley, 1987).

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NUCLEOTIDE HYDROLYSIS AND PROTEIN CONFORMATIONAL CHANGES IN *AZOTOBACTER VINELANDII* NITROGENASE IRON PROTEIN: DEFINING THE FUNCTION OF ASPARTATE 129.

LANZILOTTA, W.N. AND SEEFELDT, L.C.
Department of Chemistry and Biochemistry, Utah State University,
Logan, UT 84322-0300, USA

The biological reduction of dinitrogen catalyzed by nitrogenase requires the hydrolysis of a minimum of 16 MgATP for each N_2 reduced. The present work examines the role of a strictly conserved aspartic acid residue of nitrogenase Fe protein (FeP) in coupling MgATP hydrolysis to electron transfer and substrate reduction. The aspartic acid residue at position 129 in the *Azotobacter vinelandii* FeP has been suggested to participate in nucleotide interactions from its location in the x-ray structure near several amino acids previously identified to participate in nucleotide binding and protein conformational changes. The function of this amino acid was probed by changing aspartic acid to glutamic acid (D129E) and asparagine (D129N) by site directed mutagenesis. Characterization of the purified D129E FeP revealed a central role for Asp 129 in the nucleotide induced protein conformational changes in the FeP and possibly in the mechanism of MgATP hydrolysis. Specifically, changing Asp 129 to Glu was found to affect the communication from the MgATP binding site to the [4Fe-4S] cluster of the FeP. Data from EPR, circular dichroism spectroscopy, Fe^{2+} chelation rates, and the chemical shifts of isotropically shifted protons in the ^1H NMR spectra, all support the conclusion that Asp 129 participates in the nucleotide induced conformational changes in the FeP which are reflected in changes in the environment of the [4Fe-4S] cluster. The D129E FeP was found to bind both MgATP and MgADP with high affinity. The K_d determined for MgADP binding (K_d =131 μM) was comparable to that found for wild type FeP (128 μM). The affinity for MgATP binding was 1.6 times tighter than for wild type FeP (370 μM compared to 580 μM). The midpoint reduction potential of the [4Fe-4S] cluster was similar to that determined for the wild type FeP (-290 mV for wild type FeP and -300 mV for D129E FeP). Upon the addition of MgATP or MgADP, the midpoint potentials for both wild type and D129E FeP shifted to -430 and -440 mV, respectively. The D129E FeP was also found to bind to the molybdenum-iron protein (MoFeP) with normal affinity, although it could not support electron transfer to the MoFeP or MgATP hydrolysis upon docking.

DIMETHYLDIAZENES ARE REDUCED BY NITROGENASE. IMPLICATIONS FOR THE MECHANISM OF SUBSTRATE BINDING AND REDUCTION AT THE ACTIVE SITE.

McKENNA C. E. and SIMEONOV A. M.
Chemistry Department, University of Southern California, Los Angeles, CA 90089-0744, USA

Nitrogenase reduces N_2 to NH_3, but the mechanistic details are unclear. Initial $2e^-/2H^+$ reduction of N_2 could give an active site metal-bound N_2H_2 species: diazene (HN=NH, 1), or alternatively its tautomer, aminonitrene (H_2N-N). 1 is labile under typical enzyme assay conditions, and there is no direct evidence that it can be reduced by nitrogenase. Herein we present preliminary data comparing some interactions of A. vinelandii nitrogenase (Av1:Av2=7) with two stable, acyclic diazene analogues: trans- and cis-dimethyldiazene.

trans-Dimethyldiazene (trans-2) was reduced by nitrogenase to NH_3, CH_4, and CH_3NH_2 in a ratio of 1.0:1.2:1.3 ± 0.2 [$V_m(NH_3)$ = 16 nmol/mg-min] with K_m values 0.5-0.6 M. Reduction of trans-2 was inhibited non-competitively by CO and C_2H_2 with K_i values of 0.0008 and 0.006 atm respectively. cis-Dimethyldiazene (cis-2) (Simeonov, McKenna, 1995) was also reduced by nitrogenase to NH_3, CH_4 and CH_3NH_2 (1.0:1.0:1.6 ± 0.1; $V_m(NH_3)$ = 47 nmol/mg-min) with K_m values near 0.06 M. Cis-2 was inhibited non-competitively by CO and C_2H_2 with K_i 0.001 and 0.007 atm respectively. C_2H_2 reduction was competitively inhibited by cis-2 with K_i 0.1 M.

Similar K_m values for the three products from each isomer suggest that they arise from a common enzyme-substrate complex or intermediate. Trans-2 has a large K_m, indicating weak binding to the active site of nitrogenase. The K_m of cis-2 is 10x smaller, although >102x larger than that of N_2, pointing to steric and perhaps electronic effects in binding of the diazene substrates to the active site, but no absolute requirement for a specific orientation of their NN sp^2 lone pairs. Structural differences also affect the rate of electron transfer from enzyme to bound 2: V_m values for cis-2 are severalfold larger than those for trans-2, and an order of magnitude smaller than $V_m(N_2)$. The two isomeric substrates exhibit similar patterns of inhibition by C_2H_2. The observed product ratios are obviously inconsistent with symmetrical reduction intermediates undergoing exclusively C-N or N=N cleavage but could be explained by a mixture of these two processes. By contrast, a single reduction path with initial release of CH_4 predicts a product ratio of 1:1:1 for 100 % subsequent N=N cleavage, and <1 eq. CH_3NH_2 if significant C-N cleavage occurs.

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EPR, ENDOR AND ESEEM INVESTIGATION OF THE METAL CENTER COORDINATION ENVIRONMENT OF METAL ADENOSINE RIBO-NUCLEOTIDE COMPLEXES IN THE PRESENCE OF THE NITROGENASE IRON-PROTEIN FROM *KLEBSIELLA PNEUMONIAE*

JAN PETERSEN[†], CHRISTOF GESSNER[‡], CLAIRE J. MITCHELL[†], KARL FISHER[†], DAVID J. LOWE[†] AND WOLFGANG LUBITZ[‡]
[†]BBSRC Nitrogen Fixation Laboratory, University of Sussex, Falmer, Brighton BN1 9RQ, U.K.; [‡]Max-Volmer-Institut für biophysikalische und physikalische Chemie, TU-Berlin, Str. des 17. Juni 135, 10623 Berlin, Germany

The nucleotide binding sites of metal substituted ADP and ATP chelate complexes in the presence of the nitrogenase Fe-protein (Kp2) from *Klebsiella pneumoniae* have been characterized by EPR, ENDOR and ESEEM spectroscopy. Either paramagnetic divalent Mn^{2+} or VO^{2+} was used in stoichiometric amounts for the substitution of the naturally bound Mg^{2+}. ^{1}H, ^{31}P, ^{23}Na and probably ^{14}N superhyperfine (shf) couplings have been identified to interact with the metal center.

The ^{31}P shf-couplings have been attributed to interactions with phosphorus from the phosphate groups of the nucleotide. The ^{31}P resonances detected by ESEEM for Mn^{2+}-substituted nucleotides display a coupling which is consistent with that arising from an interaction with phosphate group(s) which coordinate the metal center ($A^{iso} \approx 4$ MHz). A small hf-anisotropy of the phosphorus coupling was identified for ADP and ATP yielding a metal-phosphorus distance of about 3.3 Å. ENDOR results for VO^{2+}-substituted nucleotides also show a ^{31}P shf-coupling but the most intense features arise from an interaction with a much less strongly coupled phosphate group. Since this shf-coupling is only detected for the ATP-bound form, but was absent for ADP, it has to be specific to the triphosphate nucleotide. This suggests that the weaker ^{31}P coupling arises from an interaction with a different phosphate group, probably the non-coordinated phosphate of ATP. The results clearly indicate that both metal centers, which were investigated in the presence of the Fe-protein, are closely associated with the nucleotide.

A shf-coupling of the divalent metal center with a monovalent ^{23}Na-cation was detected under the preparational conditions used for the ENDOR and ESEEM samples. This was observed for both Mn^{2+} or VO^{2+} and ADP or ATP complexes in the presence of Kp2. Similar experiments have been performed with preparations using K^{+}; this completely eliminated the Na^{+} resonances but had no effect on the ^{31}P signal. Whether the monovalent metal center represents a genuine cofactor, such as observed for pyruvate kinase (Mildvan et al., 1974), where it is used to orientate the substrate pyruvate, and if, what its role might be, is presently unclear.

EPR data indicate that two pH forms exist for VO^{2+} substituted nucleotides which has been attributed to the absence or presence of an axially coordinated inner-sphere hydroxyl molecule affecting the ligation structure of the metal nucleotide complex. In addition, VO^{2+} hyperfine EPR spectra are a composition of probably two VO^{2+} signals with significantly different EPR-parameter, pertinent to different ligand field strengths, which further suggests the presence of inequivalent binding sites or metal-nucleotide configurations. The metal coordination structure in the nucleotide bound Fe-protein will be compared with regard to other nucleotide binding proteins.

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IDENTIFICATION OF THE NITROGENASE FeMo-COFACTOR IN *AZOTOBACTER VINELANDII* AND *RHODOBACTER CAPSULATUS* USING THE TDPAC-TECHNIQUE

C. POHLMANN [A], H. APPEL [A], W.-G. THIES [B]
[a] *Institut für Experimentelle Kernphysik, Universität Karlsruhe, Germany*
[b] *Forschungszentrum Karlsruhe, Institut für Toxikologie, Germany*
A. MÜLLER [C], K. SCHNEIDER [C], W. SUER [C]
[c] *Lehrstuhl für Anorganische Chemie I der Universität Bielefeld, Germany*

The molybdenum binding state of the nitrogenase FeMo-cofactor can be studied using the time differential perturbed angular correlation (TDPAC-) technique. This spectroscopic method allows the identification of the FeMo-cofactor via the nuclear quadrupole interaction (NQI). The angular correlation is observed for the γ-rays emitted by the radioactive decay $^{99}Mo(\beta^-)^{99}Tc$. The TDPAC-technique provides a characteristic frequency ('finger-print') for each binding-state of the probe isotope ^{99}Mo.

Cells of two selected N_2-fixing bacteria species, *Rhodobacter capsulatus* and *Azotobacter vinelandii*, were grown in nitrogenase-derepression media containing $^{99}MoO_4^{2-}$. For both bacteria species the NQI-signal measured with the intact cells showed the same frequency ($\nu_Q \approx 70$ MHz). This frequency was also observed with the isolated MoFe-proteins obtained after DEAE-Sephacel fractionation (280 mM NaCl in 50 mM Tris/HCl, pH=7.8, 4 mM dithionite) under anaerobic, reducing conditions. However, in contrast to the signal observed with the intact cells, the NQI-signal of the isolated MoFe-proteins showed a significantly lower frequency broadening. This indicates a well-defined binding state which can therefore clearly be attributed to the molybdenum binding state in the FeMo-cofactor.

A significant higher frequency, detected with intact cells of *A. vinelandii* grown under strict repression of nitrogenase (in the presence of 20 mM NH_4^+), could be assigned to the Mo-storage protein [Pienkos and Brill, 1981]. This frequency was also observed after G25-Sephadex fractionation of the crude extract of these cells. However after DEAE-Sephacel fractionation (150 mM NaCl in 50 mM Tris/HCl, pH=7.8, 4 mM dithionite) of the crude extract, only the unperturbed TDPAC-signal was detected. This result could mean that the storage protein is not resistant towards DEAE-Sephacel fractionation and releases molybdenum as free MoO_4^{2-}.

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ALTERING THE [4Fe-4S] CLUSTER ENVIRONMENT OF *AZOTOBACTER VINELANDII* NITROGENASE IRON PROTEIN: THE ROLE OF PHENYLALANINE 135

RYLE, M.J. AND SEEFELDT, L.C.
Department of Chemistry and Biochemistry, Utah State University, Logan, UT, 84322-0300, USA

Nitrogenase consists of two separable component proteins, the iron protein (FeP) and the molybdenum iron protein (MoFeP). The FeP, with 2 MgATPs bound, binds to the MoFeP, hydrolyzes 2 MgATPs, and transfers an electron to the MoFeP. Using site directed mutagenesis, we are investigating how electron transfer is coupled to MgATP hydrolysis by altering residues near the [4Fe-4S] cluster of *A. vinelandii* nitrogenase FeP. Upon MgATP binding to the FeP, a conformational change is induced which results in a decrease in the midpoint potential from -300 to -430 mV. EXAFS studies have shown the Fe-Fe and Fe-S bond distances do not change upon MgATP binding(1), therefore the protein environment around the [4Fe-4S] cluster must control the midpoint potential. The recent crystal structure of *A. vinelandii* nitrogenase FeP reveals the [4Fe-4S] cluster has van der Waals contact with Ala 98, Val 130, and Phe 135, two from each subunit(2). We predict that by altering these residues to more hydrophilic residues, or to residues that can hydrogen bond to the cluster, FePs with more positive midpoint potentials will result(3). We have constructed and purified F135Y, F135W, and F135H altered FePs and found that these altered FePs are inactive both *in vivo* and *in vitro*. Phenylalanine 135 altered FePs have a normal [4Fe-4S] cluster as demonstrated by the presence of a rhombic $S=1/2$ EPR signal. Circular dichroism and NMR spectroscopy both indicate that the alteration of F135 affects the electronic properties of the cluster. F135 altered FePs bind both MgADP and MgATP, with an increased affinity for MgATP in the reduced state. Data from EPR, circular dichroism, and chelation studies all show that the nucleotide induced conformational changes are inhibited for F135 altered FePs. This is the first example of communication from the [4Fe-4S] center to the nucleotide binding site. Significantly, the alteration of F135 to tyrosine, tyrptophan and histidine results in FePs with -265, -259, and -198 mV midpoint potentials, respectively. Clearly, phenylalanine 135 play a role in defining the properties of the [4Fe-4S] cluster of *A. vinelandii* nitrogenase FeP.

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CARBONYL SULFIDE AND CARBON DIOXIDE AS NEW SUBSTRATES AND CARBON DISULFIDE AS A NEW INHIBITOR OF NITROGENASE.

SEEFELD, L.C. (1), RASCHE, M.E. (2) AND ENSIGN, S.A. (1).
(1) Department of Chemistry and Biochemistry, Utah State University, Logan, UT 84322-0300, USA.
(2) Department of Biochemistry and Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061.

Nitrogenase has been shown to reduce a variety of substrates in addition to N_2 and protons. General properties of alternative substrates for nitrogenase are the presence of N-N, N-O, N-C and C-C triple or double bonds. In the present work, we demonstrate that *Azotobacter vinelandii* nitrogenase can reduce both C-S and C-O bonds. Nitrogenase was found to reduce carbonyl sulfide (COS) to CO and H_2S at a maximum rate of 37.2 ± 2.0 nmoles • min^{-1} • mg $protein^{-1}$ with a K_m of 3.1 ± 0.6 mM. The formation of CO from nitrogenase reduction of COS was monitored spectrophotometrically in real-time by following the formation of carboxyhemoglobin. In this assay, the change in the visible absorption spectrum of reduced hemoglobin upon binding CO provided a sensitive way to quantify CO formation and to remove CO, which is a potent inhibitor of nitrogenase, from solution. COS reduction by nitrogenase required the molybdenum-iron protein (MoFeP), the iron protein (FeP), and MgATP. The reduction reaction was inhibited by MgADP, acetylene and N_2, while H_2 was not an inhibitor of COS reduction. The pH optimum for COS reduction was 6.5. Nitrogenase was also found to reduce carbon dioxide (CO_2) to CO and H_2O. CO_2 was reduced at a maximum rate of 0.8 ± 0.07 nmoles • min^{-1} • mg $protein^{-1}$ with a calculated K_m for CO_2 of 23.3 ± 3.7 mM. COS and CO_2 did not inhibit proton reduction by nitrogenase or total electron flow through nitrogenase. In contrast, the COS and CO_2 analog, carbon disulfide (CS_2) was found to be an inhibitor of nitrogenase reduction reactions and total electron flow. CS_2 was a rapid-equilibrium, mixed-type inhibitor with respect to acetylene reduction ($K_{i1} = 2.9 \pm 0.6$ mM and $K_{i2} = 12.1 \pm 2$ mM) and also inhibited both proton and COS reduction.

Scheme 1

$$N{=}N{=}N^- + 2e^- + 3H^+ \rightleftharpoons N{\equiv}N + H_3N$$

$$N{=}N{=}O + 2e^- + 2H^+ \rightleftharpoons N{\equiv}N + H_2O$$

$$S{=}C{=}O + 2e^- + 2H^+ \rightleftharpoons C{\equiv}O + H_2S$$

$$O{=}C{=}O + 2e^- + 2H^+ \rightleftharpoons C{\equiv}O + H_2O$$

THEORETICAL STUDY OF ELECTRONIC STRUCTURE OF THE Fe-Mo-COFACTOR AND ITS POSSIBLE COMPLEX WITH N_2

SHESTAKOV, A.F.
Institute of Chemical Physics, Russian Academy of Sciences,
Chernogolovka, Moscow Region, 142432, Russia.

In local surrounding of Fe-Mo-Cofactor, there are three residues which are able to carry positive charge; Arg96, Arg359 and His195. So from the electroneutrality principle, the group (Cys 275)Fe_7S_9Mo(His 442)(HomoCit) has total negative charge in the range 0 - (-3). Assuming variations of the homocitrate residual charge in the limits from 0 to -2, one concludes that allowed charge of Fe_7S_9Mo core is in the interval (+2) - (-3). Detail consideration shows that in the rest state of cofactor there are valencies distribution Mo^{IV} $4Fe^{II}$ $3Fe^{III}$. From this model and high symmetry of Fe-Mo-Cofactor follows only three different types of Fe atoms. One obtains total agreement with experimental data (5 types of Fe) if one suppose that Y atom in Kim and Rees structure is actually protonated S. This S atom has very short distance to polar group of Arg96, so proton transfer from Arg96 to it in nonpolar surrounding seems to be allowed. EHT calculations of C_{3v} model $(H)_3MoFe_3S_9Fe_3Fe(H)$ show that Mo levels occupy a top of d-band. Taking into account atomic Hund energy in Local Spin Hamiltonian model, one has that indeed configurations with unpaired electrons (up to achieving d^2 Mo state) result in energy gain. Supposition about large exchange between spins of Fe atoms on edges of Fe_6 prism allows to qualitatively explain cofactor spin states (rest - S=3/2, ox - S=0, red - S=1) if capped Fe atom is Fe^{III}.

Two model of N_2 binding were considered; N_2 above Fe_4 face along its diagonal and N_2 half-penetrating into cofactor through Fe_4 face. In both models, N_2 has very low overlap population of N-N bond. For the first model, transition to configuration with high local spins has great influence on the N-N bond weakening at coordination. This model gives more negative charge on N_2 molecule but short N-S contacts (with positive N-S overlap population). N_2 coordination in the second model is analogous to one in known "cage" complex $[N_2Sm_2Li_4]$.

PURIFICATION AND PROPERTIES OF NITROGENASE MoFe PROTEIN FROM A *nifZ* DELETION STRAIN OF *AZOTOBACTER VINELANDII*

ZHONG Z-P, HU C-Z, WANG J-W, HUANG J-F, LOU A-L
and LI J-G
Institute of Botany, Academia Sinica, Beijing 100044, China

The *nifZ* deletion strain of *Azotobacter vinelandii*, designated DJ194, grows poorly under N_2 fixing condition(Jacobson et al., 1989). The nitrogenase component protein activities of DJ194 showed that it contained normal levels of Fe protein activity but lower MoFe protein activity(Jacobson et al., 1989). We purified the DJ194(Δ*nifZ*) MoFe protein and studied its properties by comparing with the purified wild-type *Azotobacter vinelandii* MoFe protein. The DJ194 MoFe protein was purified according to the wild-type MoFe protein purification procedure(Burgess et al., 1980) with modifications. The heating step of the cell free extract was at 52°C for 5 min and then centrifuged. This extract was then further purified by using column chromatography, first through DEAE-cellulose 52, then Sephacryl S-200 and then finally a Q-Sepharose column. From each column the first major brown peak, which eluted from the column, was collected and loaded onto the next column. Each step of the MoFe protein purification was monitored by the SDS PAGE, Western blotting and C_2H_2 reduction activity.
The cell free extract of DJ194 is more sensitive to O_2 and heat than the wild-type extract. The purified DJ194 MoFe protein is an $\alpha_2\ \beta_2$ tetramer. The specific activity of the protein is 300nmol of C_2H_4 formed/min/mg of protein. The UV and visible absorption spectra of the $\triangle$*nifZ* MoFe protein are similar to those of the wild-type MoFe protein. But the CD and MCD spectra of the $\triangle$*nifZ* MoFe protein showed significant differences compared with the wild-type MoFe protein at the region around 450nm. This seems to indicate that the P-cluster of the $\triangle$*nifZ* MoFe protein is modified. Thus, the modification of the P-cluster in the protein may be the cause of the low activity of the DJ194 MoFe protein.

Jacobson MR et al(1989)Mol.& Gen. Genet. 219,49-57.
Burgess BK et al(1980) Biochim. Biophys. Acta. 614,196-209.

SECTION 3

REGULATION OF NITROGEN FIXATION

REGULATION OF NITROGEN FIXATION: PROGRESS AND PROSPECTS 1995

KENNEDY, C. AND SHESTAKOV*, S.
Department of Plant Pathology, University of Arizona, Tucson AZ 85721, USA and *Department of Genetics, University of Moscow, Moscow, Russia

Environmental conditions regulate the synthesis and/or activity of nitrogenase enzyme in all nitrogen fixing bacteria examined. The most significant of these environmental factors are the availability of fixed nitrogen and molecular oxygen. In addition, in organisms containing one or more alternative nitrogenases, the amounts of available molybdenum or vanadium compounds can influence nitrogenase activity and expression of *nif*, *vnf* and *anf* nitrogen fixation genes. This Symposium addressed current understanding of how ammonium, oxygen, and metals regulate nitrogen fixation in a wide range of diazotrophs.

Control of nitrogen fixation gene expression in Proteobacteria via NifA and sigmaN

A model for regulation of *nif* gene expression in *Klebsiella pneumoniae* was first presented at the 4th International Congress on Nitrogen Fixation, Canberra, 1980. Its main elements were that the transcriptional activator NtrC was necessary for expression of the *nifLA* operon and that NifA activated transcription of all other *nif* operons, while NifL prevented their transcription if oxygen was present. This model was later elaborated to include ammonium as an effector of NifL, the alternative sigma factor σ^N for recognition of *nif* promoters, and the necessity of NtrB to phosphorylate NtrC in N-limited cultures. The *K. pneumoniae* paradigm became the baseline from which questions were formulated and experiments designed to understand how *nif* genes were regulated in other diazotrophs. There are two features common to all the several genera analyzed, viz. all *nif* operons are preceded by a promoter recognized by σ^N and their transcription is activated by NifA (the only exception is expression of some operons containing *nifA*). However, the variety of mechanisms displayed among the Proteobacteria for ammonium and oxygen regulation of NifA activity and/or *nifA* gene expression is diverse and the results presented at this Symposium enrich our understanding of how the important NifA protein is available for activation of *nif* genes when nitrogen fixation is necessary and possible.

Only three genera of diazotrophs, *Klebsiella, Enterobacter* and *Azotobacter* (all members of the delta group of Proteobacteria), appear to have a *nifL* gene whose product inactivates NifA; in these organisms, *nifL* is upstream from and cotranscribed with *nifA*. Dixon et al. presented evidence indicating that *A. vinelandii* NifL is a flavoprotein that in its active form prevents the NifA-dependent conversion of *nif* promoter+RNA-polymerase complexes to the open form required for transcription. NifL is activated by nucleotides, ADP in particular, suggesting a link between energy status and regulation of nitrogen fixation. How NifL is converted to its active form in the presence of ammonium is not yet

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known, but probably involves the product of a *glnD*-like gene in *A. vinelandii* (Meletzus et al.). Unlike in *K. pneumoniae* and *E. agglomerans* (Rubie), *nifA* gene expression in *A. vinelandii* is not prevented in ammonium and does not require NtrC. Therefore NifA activity is the only target for ammonium control of nitrogen fixation in *A. vinelandii*. A final highlight of the delta group was the report by Merrick and Taylor that two of the four genes located downstream of *rpoN* in *K. pneumoniae*, *ptsN* and *ptsO*, negatively control the activity of σ^N; mutations in these genes result in higher levels of expression of three other σ^N dependent promoters.

The alpha group of Proteobacteria were represented by reports in the main symposium concerning *Rhodobacter* and *Azorhizobium*. In both of these genera, expression of *nifA* requires phosphorylated NtrC, including both of the identical copies of *nifA* in *Rhodobacter capsulatus*, and therefore *nifA* expression in these two organisms is regulated by ammonium because NtrC is only phosphorylated in the absence of fixed N. An interesting discovery in *R. capsulatus* is that expression of both *nifA* genes apparently requires an unknown sigma factor, designated σ^X, and not σ^N (Klipp et al.). Both NifA proteins are inactivated in cultures grown with high ammonium or oxygen; inactivation of NifA involves the product of the *hvrA* gene which is found in a cluster of photosynthesis regulatory genes. This is another example of an interlink between the regulatory circuits of nitrogen fixation and energy metabolism. *R. capsulatus* also contains the *anf* genes for expression of the Fe-only nitrogenase. Expression of the *anfA* gene, like *nifA*, requires NtrC-P and still another sigma factor, as yet unidentified and named σ^Y. Other details of *R. capsulatus* and *A. caulinodans* regulation, including oxygen regulation of *nifA* expression, are presented in the relevant papers by Klipp et al. and Kaminsky et al. Also of interest in this group is that the NifA protein of *Herbaspirillum seropedicae* is inactive in ammonium- or oxygen-grown cultures (Souza et al.); truncated forms of the protein missing the N-terminus are insensitive to ammonium but still regulated by O_2, as expected from the predicted O_2-sensitive interdomain linker present in the C-terminal region of NifA proteins characterized in all members of the alpha group of Proteobacteria.

The emerging importance of *glnB* in nitrogen-fixing bacteria

An unexpected highlight of the St. Petersburg meeting was the plethora and diversity of reports on the importance of the *glnB* gene product, PII, in diazotrophs and other bacteria. From the enteric model of ammonium sensing, PII is uridylylated in cells grown in low levels of fixed N. PII-UMP stimulates phosphorylation of NtrC and deadenylylation of glutamine synthetase (GS); deuridylylated PII stimulates the opposite activities. The question of whether PII is involved in NifA synthesis and activity and other responses to ammonium is an important one. In this Symposium, evidence for the presence of two *glnB* genes in *Escherichia coli* (van Heeswijk et al.) and in *Azospirillum brasilense* (de Zamaroczy et al.) was reported. Also, the Archaebacterium *Methanococcus maripaludis* carries two *glnB*-like genes interspersed with *nif* genes found in an 8 Kb gene cluster, all of which are required for nitrogen fixation (apparently Mo-dependent, despite the greater similarity of *nifH* in this cluster to the *anfH* genes of other organisms) (Kessler et al). In *E. coli*, one *glnB* gene is constitutively expressed; a second, newly discovered *glnB* gene is apparently only expressed in the absence of ammonium. In *A. brasilense*, the *glnB* gene located upstream of *glnA* (an arrangement conserved in all delta group Proteobacteria analyzed), is required for inactivation of NifA in the presence of ammonium by an unknown mechanism, but is not involved in adenylylation/deadenylylation of GS (Arsene and Elmerich). A second *glnB* gene has now been identified elsewhere in the genome of this organism; so far its function is unknown (de Zamaroczy et al.). A *glnB* gene has also been identified in *Azotobacter vinelandii*; its sequence is similar to those in other Proteobacteria including the conserved Tyr site of uridylylation by GlnD (Meletzus et al.). Mutations in this *glnB* gene are lethal when introduced into *A. vinelandii* so its role in nitrogen fixation cannot be assessed. Of interest is that the *glnB* gene of *A. vinelandii* is located upstream of an *nrgA*-like gene whose product in eukaryotes is part of a high-affinity ammonium transport process. Finally, mutations in *glnB* genes of *R. meliloti* or *R. leguminosarum* bv. *vicia*, located upstream of *glnA* as in other members of the delta group of Proteobacteria, result in strains that are symbiotically defective in nodule morphogenesis and result in early senescence of bacteroids (Arcondeguy et al., Fedorova et al.).

Regulation of nitrogenase activity

Both molybdenum and alternative nitrogenases are regulated at the post-translational level by one or more mechanisms in response to ammonium, oxygen supply and light limitation in photosynthetic bacteria. The first system is associated with reversible modification of dinitrogenase reductase by ADP-ribosylation. This system is determined by the products of *draT* and *draG* genes which are also found in *A. brasilense*. DRAT and DRAG activities are regulated in vivo but not by a simple ON/OFF switch. DRAT is involved in the control of DRAG activity (Ludden et al.). Also, the darkness and anaerobiosis signals are distinct from the fixed nitrogen signal and the concentration of NAD^+ appears to be important in regulating DRAT and DRAG activities. Both nitrogen and redox states are therefore significant for regulation of DRAT and DRAG (Noren and Nordlund). Photosynthetic bacteria and some Cyanobacteria may also have other systems for reversible inactivation of nitrogenase in response to ammonium and oxygen. These regulatory systems appear not to involve ADP-ribosylation (Yakunin, Hallenbeck) but their mechanisms and genetic basis remain to be elucidated.

Also in the context of nitrogenase activity were reports that analysis of nitrogenase-linked hydrogen evolution have led to the identification of new oxidoreductase gene (Bothe et al) and a second Mo nitrogenase in *Anabaena* (Schrautemeier et al).

Outlook for the future

de Bruijn et al. reported that *lux* gene reporter fusions can be used to identify genes in *R. meliloti* that are differentially expressed in response to fluxes in fixed N, carbon, oxygen, or plant root exudate supply. This method should allow the identification of genes not only in species of *Rhizobium*, but also in other symbiotic, associative, or free-living diazotrophs that respond to environmental fluxes and might be important for nitrogen fixation efficiency or effectiveness. In addition, the use of a novel reporter system, the green fluorescent protein, is allowing regulation of cell differentiation in filamentous Cyanobacteria to be analyzed. Both ammonium and oxygen prevent heterocyst formation. Functions for *hetR* and other genes were reported by Haselkorn. We look forward to applications of these and other methods to reveal genes in addition to those so far identified that are important for regulation of nitrogen fixation.

We predict that at the 11th International Congress on Nitrogen Fixation, great progress will be reported on the roles of the ammonium response regulatory proteins PII and GlnD in various aspects of nitrogen fixation or nodulation. We will learn about the possibile duplication of *glnB*-encoded PII proteins in various diazotrophs and how each contributes to nitrogen fixation and/or ammonium assimilation. From the reports at this meeting, we know or predict that the *glnB* and *glnD* genes encoding the PII and GlnD proteins may be involved in modulating the activities of NifA and NifL proteins, glutamine synthetase, NtrB and NtrC, and possibly ammonium transport. As in the past, the future will show a great diversity of mechanisms by which environmental factors modulate nitrogen fixation through control of gene expression or nitrogenase activity. By understanding these control mechanisms, strategies for increasing nitrogen fixation capabilities in free-living, associative, and symbiotic nitrogen fixing bacteria will evolve.

REGULATION OF NITROGEN FIXATION BY THE NIFL AND NIFA PROTEINS FROM *AZOTOBACTER VINELANDII*

DIXON R., AUSTIN S., EYDMANN T., JONES T., SÖDERBÄCK E.
AND HILL S.
Nitrogen fixation laboratory, University of Sussex, Brighton, BN1 9RQ, UK

INTRODUCTION

The extreme oxygen sensitivity of the nitrogenase component proteins and the high energetic requirements for nitrogen fixation impose physiological constraints on diazotrophy necessitating stringent regulation of *nif* gene transcription in response to fixed nitrogen and environmental oxygen (Hill, 1992). In most diazotrophs expression of genes required for the synthesis of molybdenum nitrogenase is positively controlled by the transcriptional activator NIFA, an enhancer binding protein which catalyses the formation of open promoter complexes by the holoenzyme form of RNA polymerase containing the alternative sigma factor σ^N ($E\sigma^N$) (Morett and Segovia 1993). Binding sites for NIFA, termed Upstream Activator Sequences (UAS) are usually located more than 100 base pairs upstream of the transcription start site and the activator appears to make contact with downstream bound $E\sigma^N$ via the formation of a DNA loop (Buck et al 1987). The formation of such loops is facilitated by the binding of an auxilliary protein, Integration Host Factor (IHF) which may stabilise these structures by correctly orientating the activator and polymerase binding sites and offsetting the bending energy required for DNA loop formation (Hoover et al 1990). Productive interactions between NIFA and $E\sigma^N$ promote the transition from the closed promoter complex to the open promoter complex. An unusual feature of this reaction with respect to prokaryotic transcription systems is that it requires the hydrolysis of a nucleoside triphosphate, principally ATP or GTP (Popham et al 1989; Berger et al 1994; Austin et al 1994) (Fig 1.)

An additional regulatory protein, NIFL, controls NIFA activity in *Azotobacter vinelandii* and *Klebsiella pneumoniae* in response to oxygen and fixed nitrogen. Sequence analysis indicates that *A.vinelandii* NIFL is comprised of two domains: the amino terminal domain appears to be sensory and shows homology to other oxygen responsive regulatory proteins including FixL and Bat whereas the C-terminus resembles the conserved transmitter domain found in members of the histidine protein kinase

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family of two-component regulatory proteins (Blanco et al 1993). Although *A.vinelandii* NIFL possesses all five of the conserved regions found in other transmitter domains as well as the conserved histidine residue known to be the autophosphorylation site in other members of this family, many substitutions in this histidine residue do not impair NIFL function and therefore sensory transduction by this protein does not apparently involve histidine autophosphorylation (Woodley et al 1994). Moreover, neither autophosphorylation of NIFL nor phosphotransfer from NIFL to NIFA have so far been detected *in vitro*, suggesting that NIFL is not a *bona fide* histidine protein kinase. In this review we report recent data from our laboratory on the characterisation of *A.vinelandii* NIFL and its ability to modulate transcriptional activation by NIFA in response to both redox and energy status.

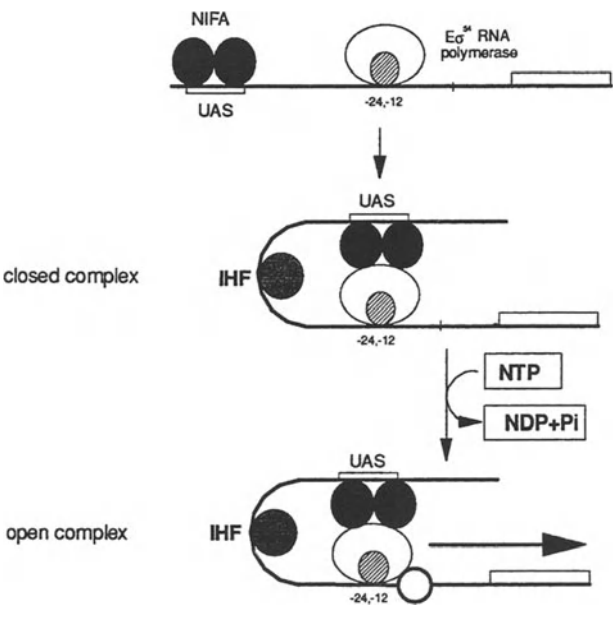

Figure 1 Pathway of transcriptional activation by NIFA

A.VINELANDII NIFL IS A FLAVOPROTEIN

During purification of NIFL under aerobic conditions we noticed that both the native protein and a modified form of NIFL containing six histidine residues at the C-terminus, (NIFL-$_{6his}$) were yellow in colour indicative of a chromophore. Visible spectra revealed an absorption maximum of 445nm suggestive of a flavin species, with additional shoulders at 420 and 470nm indicative of protein bound moeity (Fig 2). Identical spectra were obtained independent of purification method.

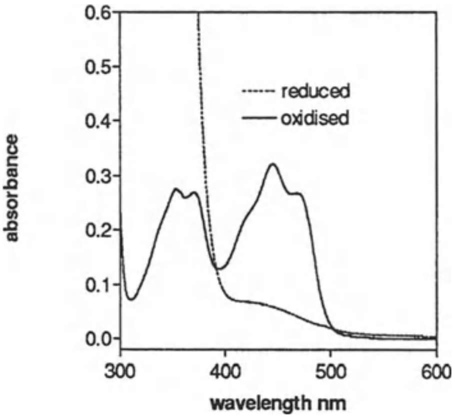

Figure 2 Visible adsorption spectra of oxidised *A.vinelandii* NIFL-$_{6his}$ and the reduced form after treatment with sodium dithionite.

Following denaturation of NIFL and analysis of the supernatent solution by TLC, a major fluorescent species with an R_f characteristic of FAD was identified. The behavior of NIFL on gel filtration chromatography suggests that it is a tetramer and the extinction coefficient indicates an FAD content of ~ 2 per mol.

NIFL MODULATES NIFA ACTIVITY VIA A REDOX SENSITIVE SWITCH

The absorption maximum of the NIFL flavin moiety was bleached upon reduction with sodium dithionite (Fig 2) and reappeared upon oxidation in air. We therefore anticipated that the redox state of the flavin may influence NIFL activity. Since NIFL itself does not have any known catalytic function it is necessary to measure its ability to inhibit trancriptional activation by NIFA in order to assay its activity. The effect of NIFL on the formation of open promoter complexes by NIFA at the *nifH* promoter was measured in the presence or absence of sodium dithionite. To ensure that NIFL was maintained in the reduced form these experiments were carried out in an anaerobic glove box (<1 ppm O_2). Reactions were perfomed on linear DNA templates in the presence of $E\sigma^N$, IHF and GTP (Fig1). Promoter complexes which have undergone the transition from the closed to the open complex are resistant to heparin challenge and were quantitated using a gel shift assay (Eydmann et al 1995). In the absence of NIFL, sodium dithionite has little effect on the activity of NIFA indicating that it did not strongly effect the ability of NIFA to catalyse open complex formation. However when NIFL-$_{6his}$ was present in the oxidised form the formation of open promoter complexes was strongly inhibited (Fig 3). In contrast when NIFL-$_{6his}$ was reduced with sodium dithionite, NIFA activity was stimulated two-fold in the later stages of the time course. (The lag phase in this curve may be due to the relatively slow rate of reduction of NIFL by dithionite). Control experiments indicated that NIFL-$_{6his}$ did not influence the activity of the related enhancer binding protein NTRC, either in the presence or absence of sodium dithionite. NIFL therefore specifically

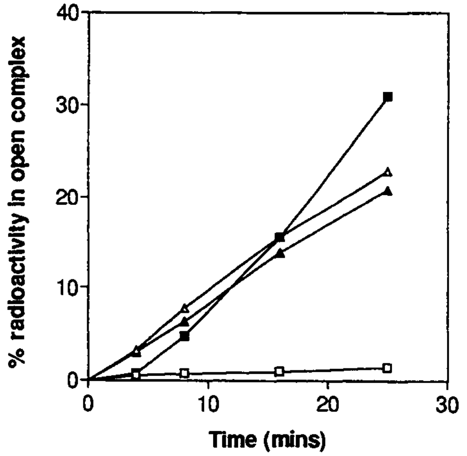

Fig 3 . Influence of redox status on inhibition of NIFA activity by NIFL. Reactions were initiated at time zero by the addition of either NIFA alone (triangles) or NIFA plus NIFL-$_{6his}$ (squares). Reaction mixtures were incubated anaerobically either in the presence (closed symbols) or absence of sodium dithionite (open symbols). Samples were taken at the indicated time intervals, heparin was then added and the reactions were analysed for the presence of open promoter complexes using the gel retardation assay and were quantitated with a phosphoimager. See Table 1 for further details of assay components.

modulates the activity of NIFA and a major switch in NIFL activity is observed when NIFL$_{-6his}$ is converted from the oxidised to the reduced form indicating that NIFL responds directly to redox status.

INFLUENCE OF ADENOSINE NUCLEOTIDES ON NIFL ACTIVITY

We have recently demonstrated that the inhibition of NIFA activity by NIFL under aerobic conditions is stimulated by the presence of adenosine nucleotides, particularly ADP. Although NIFL inhibits NIFA activity in the presence of ATP, we suggested that this inhibition could be a consequence of the formation of ADP by the catalytic activity of NIFA. Accordingly when we ensured that the ADP formed in the reaction was rapidly converted to ATP by addition of an ATP generating system, inhibition by NIFL was not observed. (Eydmann et al 1995). NIFL may therefore be responsive to the ATP/ADP ratio *in vivo*.

When ADP was added to anaerobic reactions (<1 ppm O_2) containing NIFA and GTP, NIFL$_{-6his}$ strongly inhibited open complex formation by NIFA irrespective of whether sodium dithionite was present (Table 1). Control experiments showed that ADP inhibited open complex formation by NIFA to a maximum of 34% but inhibition increased significantly (> 98%) when both NIFL$_{-6his}$ and NIFA were present. The presence of ADP does not appear to prevent reduction of the flavin moiety in NIFL, since pre-incubation of NIFL with ADP does not prevent bleaching of the 454 nm signal by sodium dithionite. Thus ADP is a major effector of NIFL activity since it overides the ability of NIFL to respond to redox status.

Table 1 Influence of ADP on the activity of oxidised and reduced NIFL

	% radioactivity in open complex[a]	
Additions[b]	**GTP only**[c]	**GTP + ADP**[d]
NIFA only	22.9	15.1
NIFA + dithionite	20.9	16.0
NIFA + NIFL	1.5	0.4
NIFA +NIFL + dithionite	40.0	0.4

[a] Reactions were carried out in an anaerobic glove box and quantitated as the percentage of total radioactivity in heparin resistant open complexes separated on native gels.

[b] Final concentration of NIFA and NIFL$_{-6his}$ was 200nM and 400nM respectively (calculated as the dimer forms).

[c] Final concentration 4mM.

[d] Final concentration 3.95 mM GTP plus 0.05mM ADP

NIFL INHIBITS THE CATALYTIC ACTIVITY OF NIFA

The mechanism of inhibition of NIFA activity by NIFL could involve a number of steps in the pathway of transcriptional activation including inhibition of specific DNA binding by the activator, interference with protein-protein contacts between NIFA and $E\sigma^N$ or inhibition of nucleoside triphosphate binding or hydrolysis by the activator. Inhibition at any of these stages suggests specific interaction between NIFL and NIFA since NIFL does not prevent transcriptional activation by the related σ^N-dependent activator NTRC. We find that *A.vinelandii* NIFA has nucleoside triphosphatase activity and that the rate of hydrolysis of ATP is similar to that of GTP. In contrast, purified NIFL has no detectable ATPase or GTPase activities. Addition of oxidised NIFL inhibited the ATPase activity of NIFL coincident with a decrease in the formation of heparin-resistant open complexes (Fig 4). Thus *A.vinelandii* NIFL can modulate the activity of NIFA by inhibiting its catalytic activity. However this might not be the sole target for NIFL inhibition. Experiments with *K.pneumoniae* NIFL and NIFA indicate that renatured NIFL inhibits transcriptional activation by the isolated central domain of NIFA in the absence of detectable inhibition of its ATPase activity. In this case the inhibitory form of NIFL may block productive interactions between NIFA and $E\sigma^N$ (Berger et al 1994).

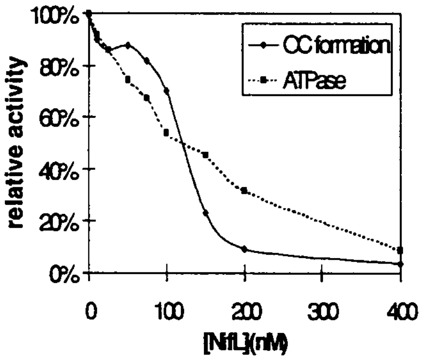

Fig 4. Inhibition of the ATPase activity of NIFA by NIFL. Both open complex formation (solid line) and ATPase activity (dotted line) were measured in the same reaction mixture.

CONCLUSIONS

The mechanism whereby NIFL signals changes in extracellular oxygen concentration to the transcriptional activator NIFA is one of the major questions concerning regulation of nitrogen fixation. The finding that NIFL is a flavoprotein suggests that its activity could be subject to changes in redox status congruent with the observation that the flavin signal is bleached by sodium dithionite and a major switch in activity is detected when oxidised NIFL is converted to the reduced form. Although the FAD moeity undoubtedly confers redox properties we cannot rule out the possibility that NIFL contains additional redox active groups. Although similarity to the sensory domain of FIXL could implicate the presence of heme (Blanco et al 1993; Gillez-Gonzales et al 1994), we find that NIFL is not directly responsive to changes in oxygen concentration (data not shown) but it clearly can directly sense the redox status. NIFL is therefore another representative of the redox-responsive class of transcriptional regulators. Since we have not detected a catalytic activity for NIFL it may represent a novel class of flavoprotein in which electron transfer is not coupled to reduction of an obvious substrate. In common with other redox-sensing regulatory proteins, changes in redox status may be linked to conformational changes which modulate NIFL activity.

In addition to acting as a redox sensor, our data suggest that NIFL may also be directly responsive to the energy status since ADP potentiates the form of NIFL which inhibits NIFA. ADP is a potent effector of NIFL activity even when the flavin moeity is fully reduced. This suggests that energy status is likely to be a principal factor in detemining whether or not nitrogenase is synthesised since it overides the influence of the redox state of NIFL. The bell-shaped response of nitrogenase synthesis to changes in O_2 supply in *Azotobacter chroococcum* appears to support this suggestion. The level of synthesis of nitrogenase polypeptides is low under an O_2 limitation, conditions which should correspond to a deprived energy status where reducing power is likely to be in excess. Nitrogenase synthesis increases as the O_2 input is raised but declines at high rates of input, where presumably there is a dearth of reducing power but an abundance of ATP (Postgate et al 1981). Our working hypothesis is that the C-terminal domain of NIFL binds adenosine nucleotides and has a greater affinity for ADP compared with ATP. Such binding may induce a conformational change in NIFL (see Söderbäck et al this volume), switching it into an inhibitory form. An analogous change in conformation may occur when the flavin moiety is oxidised.

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POSTTRANSLATIONAL REGULATION OF NITROGENASE ACTIVITY BY REVERSIBLE ADP-RIBOSYLATION; HOW ARE THE REGULATORY ENZYMES DRAT AND DRAG REGULATED?

ZHANG, Y.P.[1,2,3], GRUNWALD, S.K.[1,3], LIES, D.[2,3], HALBLEIB, C.[1,3], MA, Y.[1,3], ROBERTS, G.P.[2,3], BURRIS, R.H.[1,3] LUDDEN, P.W.[1,3]
Departments of Biochemistry[1] and Bacteriology[2] and Center for the Study of Nitrogen Fixation[3], University of Wisconsin, Madison, WI 53706 USA.

Nitrogenase activity in *Rhodospirillum rubrum, Azospirillum brasilense* and related bacteria is regulated by the reversible ADP-ribosylation of arg-101 of the dinitrogenase reductase protein (Ludden and Roberts 1989). Only one of the two arg-101 residues of the NifH homodimer is modified at one time, presumably because of steric hindrance. The ADP-ribosylation of dinitrogenase reductase prevents its productive association with dinitrogenase and thus neither electron transfer nor ATP hydrolysis occurs (Murrell et al. 1988). The NAD-dependent ADP-ribosylation of dinitrogenase reductase is catalyzed by DRAT (Dinitrogenase Reductase ADP-ribosyl Transferase) and the removal of ADP-ribose is catalyzed by DRAG (Dinitrogenase Reductase Activating Glycohydrolase). DRAT and DRAG are encoded by the *draTG* operon, which is not co-regulated with the *nif* genes but which is found near the *nifHDK* operon in *R. rubrum, A. lipoferum* and *A. brasilense* (Fitzmaurice et al. 1989; Fu et al. 1989; Fu et al. 1990; Zhang et al. 1992). Both DRAT and DRAG activities are regulated *in vivo*. Regulation of DRAG was established when ^{32}P-labelled ADP-ribose could not be chased from dinitrogenase reductase during conditions favoring modification (Kanemoto and Ludden 1984). If DRAG was unregulated, the labelled ADP-ribose group would have been continuously replaced (chased) by unlabelled ADP-ribose. tDRAT is regulated *in vivo* becuase a mutant lacking DRAG but containing DRAT activity accumulated dinitrogenase in the active form until a signal (darkness or ammonium) was given to initiate the modification (Liang et al. 1991). The regulation of both DRAT and DRAG is through inhibition of their activities by other factors, because both enzymes are active in the purified form and require no other factors for activation.

A speculative model for the regulation of DRAT and DRAG (Figure 1) shows DRAG (represented by the diamond with 2 Mn) regulated by association with DRAT (represented by the triangle). DRAG is activated by release from the complex when DRAT binds a low molecular weight signal compound(s) that has yet to be identified. When not associated with DRAG, DRAT binds to dinitrogenase reductase (Rr2). At normal levels of DRAT this does not cause inhibition, because dinitrogenase reductase is present at approximately 1000-fold higher concentration than DRAT. When the inhibition of DRAT by the signaling compound ceases, DRAT modifies dinitrogenase reductase and is then released. Free DRAT then either forms an association with another molecule of dinitrogenase reductase and modifies it or forms a complex with DRAG, in which both DRAT and DRAG are inactive. Regulation is proposed to

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result from non-covalent associations of DRAT, DRAG and effector molecules, because the regulation is lost upon extraction of DRAT and DRAG from the cell and no evidence for covalent modification(s) has been obtained by analysis of extracts on 2D gels.

Model for the regulation of DRAT and DRAG

Figure 1

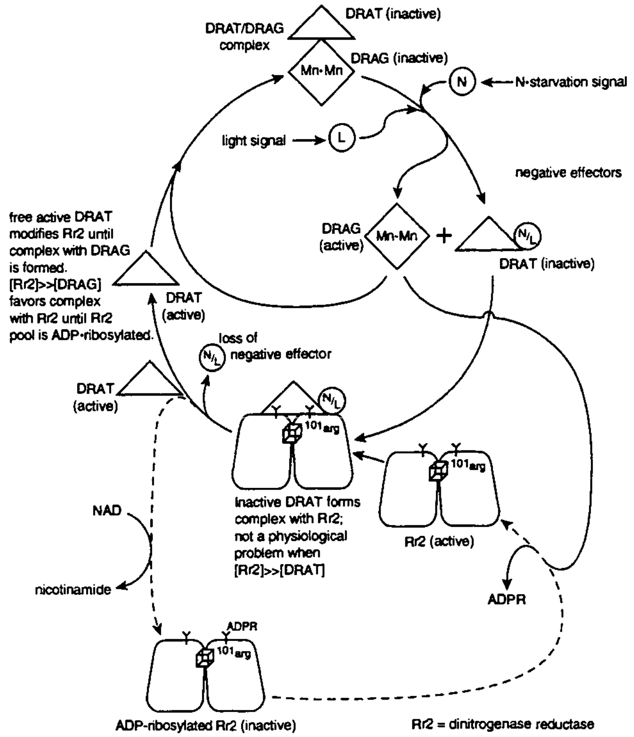
DRAT/DRAG complex
DRAT (inactive)
Mn-Mn
DRAG (inactive)
N
N-starvation signal
light signal
L
negative effectors
free active DRAT modifies Rr2 until complex with DRAG is formed. [Rr2]>>[DRAG] favors complex with Rr2 until Rr2 pool is ADP-ribosylated.
DRAG (active)
Mn-Mn
+
N/L
DRAT (inactive)
DRAT (active)
loss of negative effector
N/L
DRAT (active)
101arg
Inactive DRAT forms complex with Rr2; not a physiological problem when [Rr2]>>[DRAT]
101arg
Rr2 (active)
NAD
nicotinamide
ADPR
ADPR
101arg
ADP-ribosylated Rr2 (inactive)
Rr2 = dinitrogenase reductase

Table 1 shows the in vivo activities of DRAT, DRAG and dinitrogenase reductase under different conditions of switch-off. Upon treatment of *R. rubrum* cells with darkness or ammonium, DRAT is transiently activated and DRAG becomes inactive. DRAT is only transiently activated because dinitrogenase reductase is never completely inactivated in response to darkness or ammonium or any other stimulus studied. Even in a *draG* mutant of *R. rubrum*, 5-10% of dinitrogenase reductase remains active and unmodified; thus in the absence of DRAG activity, some component of the regulatory cycle stops DRAT activity before all dinitrogenase reductase is modified. Further, in a *draG* mutant of *A. brasilense* there are conditions where anaerobiosis leads to modification of ca. 70% of the dinitrogenase reductase; a subsequent treatment with ammonium modifies the whole dinitrogenase reductase pool. Thus, the capacity to activate DRAT to a greater extent exists in the cell, but a feature of the regulatory scheme prevents that capacity from being realized under all circumstances and it appears that DRAT is transiently activated following the signal. DRAG also is inactive under these conditions, because nitrogenase activity is not recovered, but rather a stable, partially ADP-ribosylated pool of dinitrogenase reductase is observed as long as conditions remain unchanged.

Table 1. DRAG, DRAT and Dinitrogenase Reductase Activities

CONDITION	DRAG Activity	DRAT Activity	Dinitrogenase Reductase
nif-derepressing	active	inactive	active (not ADPR)
following Dark or NH_4^+	loses activity	becomes active	becomes inactive) (ADPR)
long after Dark or NH_4^+	inactive	inactive	remains inactive* (ADPR)

*a small portion of dinitrogenase reductase remains active

Effects of Overexpression of DRAT and DRAG.

Strains of *R. rubrum* in which either DRAT or DRAG or both enzymes have been overexpressed from the *nif* promoter were constructed (Grunwald et al. 1995). In each case, the chromosomal copy of the *draTG* operon was intact and functional. When DRAG is overexpressed, the cells accumulate normal levels of nitrogenase proteins and exhibit normal levels of in vivo nitrogenase activity. The DRAG overexpresser fails to ADP-ribosylate dinitrogenase reductase in response to either darkness or ammonium, and thus the overexpressed DRAG escapes regulation. When DRAT is overexpressed, the cells accumulate approximately half the nitrogenase proteins of wild-type cells and exhibit only 5-10% of the normal, *in vivo* nitrogenase activity. The DRAT activity is appropriately regulated, as the nitrogenase activity responds to both darkness and ammonium. The low in vivo nitrogenase activity in the DRAT overexpresser is postulated to result from an interaction of DRAT with dinitrogenase reductase. In these cells, DRAT and dinitrogenase reductase are present at approximately equal concentrations and by binding to dinitrogenase reductase, DRAT interrupts the flow of electrons to dinitrogenase.No inhibition of nitrogenase activity by excess DRAT occures *in vitro*, perhaps because *in vitro* assays are performed with excess ATP and no ADP and DRAT activity is far greater with the ADP:dinitrogenase reductase complex (Lowery and Ludden 1989). When both DRAT and DRAG are overexpressed, relatively low in vivo activities are again observed even though near normal levels of nitrogenase proteins accumulate in the cell. The nitrogenase activity in the DRAT and DRAG overexpresser is regulated by ADP-ribosylation in response to darkness and ammonium. Thus regulation of DRAG, which was lost when DRAG was overexpressed in a cell with normal levels of DRAT, is regained when DRAT is overexpressed to the same level. This result is interpreted as indicating a role for DRAT in the regulation of DRAG (Figure 1.)

Multiple pathways of signal transduction leading to ADP-ribosylation of dinitrogenase reductase.

A variety of physiological conditions lead to ADP-ribosylation of dinitrogenase reductase. In *R. rubrum* these include darkness, treatment with fixed nitrogen and aerobic treatment (Neilson and Nordlund 1975; Kanemoto and Ludden 1984). In the microaerobic organism *A. brasilense*, both fixed nitrogen and anaerobiosis lead to ADP-ribosylation (Hartmann et al. 1986; Burris et al. 1991). It is now clear that the pathways regulating ADP-ribosylation can be separated so that one stimulus leads to accumulation of ADP-ribosylated dinitrogenase reductase, while another does not. With *A. brasilense*, mutations in *ntrBC* render the organism unable to respond to ammonium, whereas anaerobiosis leads to the accumulation of ADP-ribosylated dinitrogenase reductase (Fig 2) (Zhang et al. 1994). In *R. rubrum* similar results are obtained with *ntrBC* mutants; the cells respond normally to darkness but poorly to ammonium.

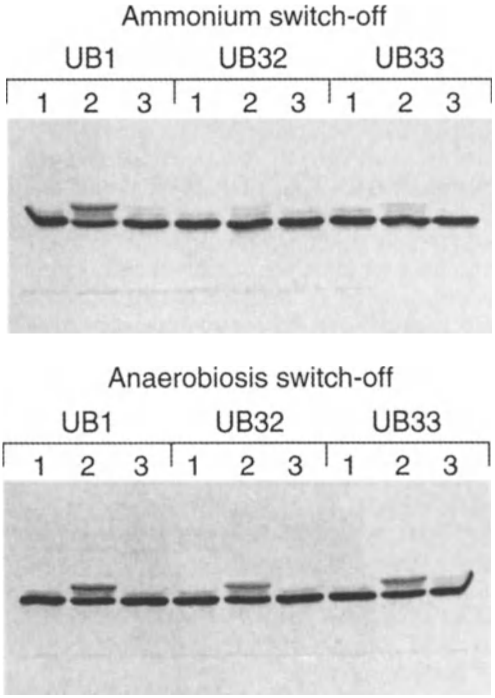

Figure 2. Effect of ammonium (top) and anaerobiosis (bottom) on the modification of dinitrogenase reductase in wild-type (UB1), an *ntrC* mutant (UB32) and an *ntrB* mutant (UB33) of *A. brasilense*. Lane 1, before treatment; lane 2 during treatment; lane 3, after treatment. The figure shows immunoblots of SDS PAGE gels of extracts. Samples treated with ammonium were exposed to 250 uM ammonium chloride for 5 minutes before sample was collected and extract prepared. Samples shown in lanes 3 were taken 15 minutes after ammonium treatment. Sample for aerobic treatment shown in lanes 2 were taken 10 minutes after initiation of aerobic treatment; samples shown in lanes 3 (post aerobic treatment) were taken 10 minutes after return to microaerobic conditions.

The inability to accumulate dinitrogenase reductase in the ADP-ribosylated form could result from an inability to release the inhibition of DRAT or an inability to inhibit DRAG. To address this question, *ntrB draG* and *ntrC draG* double mutants were prepared in *A. brasilense*. In these strains, treatment with either anaerobiosis or ammonium leads to the ADP-ribosylation of dinitrogenase reductase. From these results, we conclude that DRAT is able to function and that the defect in the *ntrBC* mutants is an inability to inhibit DRAG activity. Glutamine synthetase activity is decreased to less than 50% of normal levels in *ntrBC* mutants of *R. rubrum* and immunoblots of extracts indicate that the concentration of glutamine synthetase protein is significantly lower than in wild-type cells. We know from Nordlund's work that the *glnA* gene is cotranscribed with the *glnB* gene. Thus the concentration of the PII protein may be low in these cells as well. It is not known how the *ntrB*, *ntrC*, *glnA* or *glnB* products might interact with DRAT or DRAG, but roles for these in the regulation of ADP-ribosylation would not be surprising.

Dinitrogenase reductase plays a role in the signal transduction pathway leading to its ADP-ribosylation.

R. rubrum contains both the *nif*-encoded molybdenum nitrogenase and the *anf*-encoded Fe-only nitrogenase (Lehman and Roberts 1991). The dinitrogenase

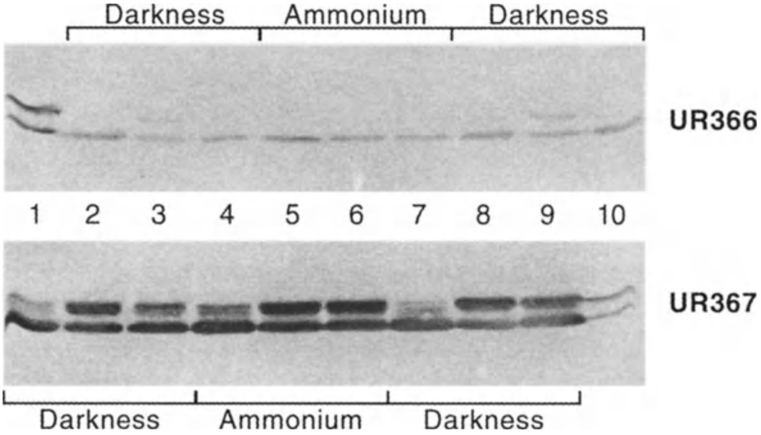

Figure 3. Effects of darkness and ammonium on the modification status of *R.rubrum* strains expressing the anf-encoded, alternative dinitrogenase reductase (UR366, top) and the *A. brasilense nifH* (UR367, bottom). The figure shows an immunoblot of an SDS PAGE of extracts of cells treated with darkness or 250 uM ammonium chloride. Top panel: Lane 1, std dinitrogenase reductase; lanes 2, 8, at the onset of darkness; lanes 3, 9 after 15 minutes of darkness; lanes 4, 10, ten minutes after return to light; lane 5, at the time of NH_4Cl addition; lane 6 after 15 minutes of NH_4Cl treatment; lane 7, after 30 minutes of NH_4Cl treatment. Bottom: lanes 1,7 at the onset of darkness; lanes 2,8 after 15 minutes of darkness; lanes 3,9 ten minutes after return to light; lanes 4,5,6 - as lanes 5,6,7 for top panel; lane 10, dinitrogenase reductase std. The upper band represents the ADP-ribosylated subunit.

reductase proteins of both nitrogenase systems are targets for ADP-ribosylation by DRAT, as they are in *Rhodobacter capsulatus* (Schneider et al. 1991).

Surprisingly, the dinitrogenase reductase of the iron-only system is ADP-ribosylated in response to darkness, but not with ammonium (Figure 3). Because DRAT ADP-ribosylates the *nif*-encoded dinitrogenase reductase in response to both, some feature of the dinitrogenase reductase apparently determines whether DRAT will modify the protein. Thus, dinitrogenase reductase plays a role in the signal transduction pathway leading to its own ADP-ribosylation.

Effect of amino acid substitutions in dinitrogenase reductase on its ADP-ribosylation.

Amino acid substitutions in dinitrogenase reductase that affect its ability to bind to dinitrogenase also affect its ability to be ADP-ribosylated by DRAT (Lowery et al. 1989) . Arginine-101 is the site of ADP-ribosylation of dinitrogenase reductase, is near the Fe_4S_4 center of the protein (Hausinger and Howard 1983; Georgiadis et al. 1992), and is part of the interaction site of the two nitrogenase components. Seefeldt and coworkers have introduced substitutions in this region of the dinitrogenase reductase from *Azotobacter vinelandii*, and they have tested these for productive complexation with dinitrogenase. The K143Q dinitrogenase reductase supports low nitrogenase activity and is able to be ADP-ribosylated at about 40% of the rate of the wild-type protein. The R140Q dinitrogenase reductase is unable to form a complex with dinitrogenase and exhibits only a slight ability to be ADP-ribosylated. These results support the hypothesis that DRAT and dinitrogenase interact with the same region of dinitrogenase reductase.

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REGULATION OF FREE-LIVING AND SYMBIOTIC NITROGEN FIXATION IN *AZORHIZOBIUM CAULINODANS*

KAMINSKI, P.A., MICHEL-REYDELLET, N., DESNOUES, N. AND ELMERICH, C.
Unite de Physiologie Cellulaire, URA 1300 CNRS, Departement des Biotechnologies, Institut Pasteur, 28 rue du Dr. Roux, 75724, Paris Cedex 15 France.

Introduction

Azorhizobium caulinodans ORS571, isolated from stem nodules of *Sesbania rostrata*, can fix nitrogen in the free-living state (Nif$^+$) and symbiotically (Fix$^+$) (Elmerich et al. 1982; Dreyfus et al. 1988). Signals involved in the regulation of nitrogen fixation are oxygen and fixed nitrogen (Ratet et al. 1989). This regulation is mediated by two-component regulatory systems which involve FixLJ, NtrBC and NtrYX (Pawlowski et al. 1987, 1991; Kaminski et al. 1991). Nif$^-$, Fix$^-$ mutant strains impaired in the synthesis of polypeptides normally induced under nitrogen free-conditions, including nitrogenase polypeptides, have been characterized (Denefle et al. 1987). This led to the discovery of two new regulatory genes. One belongs to the *lysR* family, the other shares similarity with the *E. coli hfQ* gene (Kaminski et al. 1994).

nif and *fix* genes in *A. caulinodans*

Genomic libraries were screened for genetic complementation of Nif$^-$ mutants and heterologous probes were used for hybridisation to identify the cloned genes. Various regions involved in free-living and symbiotic nitrogen fixation were thereby identified, as schematised in Figure 1. Regions I and II contain genes involved in the synthesis and function of the nitrogenase, and which are under the control of the transcriptional activator NifA (Pawlowski et al. 1987; Kaminski et al. 1988; Arigoni et al. 1992). They include the *fixABCX* genes first identified in *R. meliloti* as a symbiotic nitrogen fixation region. These genes were essential for nitrogenase activity in *A. caulinodans* in the free-living state. FixA and B resemble the α and β subunits of electron transport protein, FixX encodes a ferredoxin-like protein (Arigoni et al. 1991). The expression of *fixABCX* in *A. caulinodans* involved mRNA processing such that differential expression of the genes was possible (Arigoni et al. 1992).

Regions III and V were identified by hybridization with DNA probes obtained from the symbiotic *fix* cluster of *R. meliloti*. The translation products of *fixNOQP* and the analysis of the cytochrome content of membrane fractions suggested that this operon encodes a terminal oxidase complex, involved in a respiratory process under microaerobiosis (Mandon et al. 1994). Inactivation of the genes in the *fixNOQP* and *fixGHI* (Mandon et al. 1993, 1994) regions led to a partially reduced nitrogenase activity both *in planta* and *ex planta*, in contrast to *B. japonicum* and *R. meliloti* (Preisig et al. 1993) which were

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Fix. This suggests that the genes are not specific for symbiotic nitrogen fixation in A. *caulinodans*. Indeed, there is an additional terminal oxidase which functions in microaerobiosis, corresponding to a cytochrome *bd* complex, encoded by the *cydAB* genes (Kitts, Ludwig 1994). Region IV contains a new regulatory gene, *nrfA*, required for *nifA* expression (Kaminski et al. 1994).

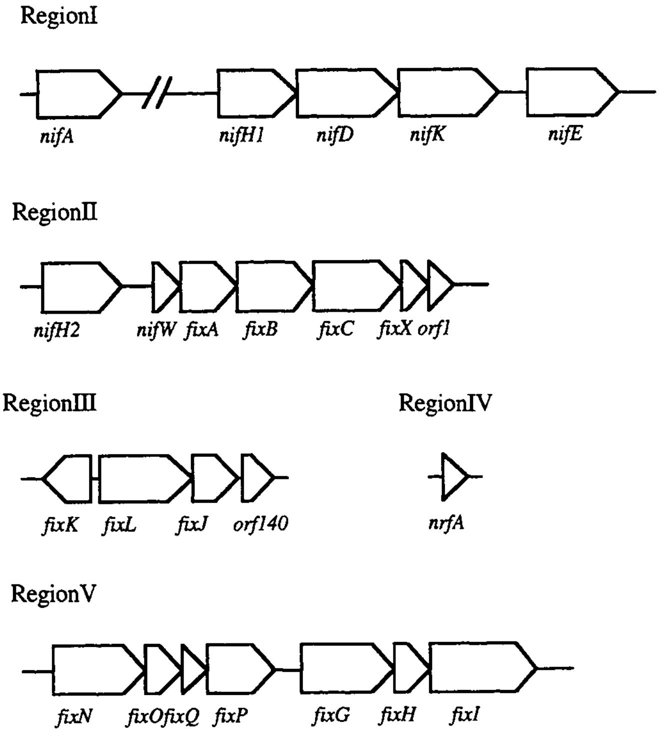
RegionI
nifA
nifH1
nifD
nifK
nifE
RegionII
nifH2
nifW
fixA
fixB
fixC
fixX
orf1
RegionIII
RegionIV
fixK
fixL
fixJ
orf140
nrfA
RegionV
fixN
fixO
fixQ
fixP
fixG
fixH
fixI

Figure1: *nif* and *fix* genes of *A. caulinodans*.

Control by oxygen: involvement of *fixLJK*.

A. caulinodans homologues of *R. meliloti fixL*, *fixJ* and *fixK* have been characterized (Kaminski, Elmerich 1991; Kaminski et al. 1991). ORF140 is immediately downstream from *fixJ* and is a truncated *fixJ*-like gene lacking the helix-turn-helix domain. Strains carrying mutations in *fixLJK* are impaired in nitrogen fixation both in the free-living and symbiotic states (Kaminski, Elmerich 1991; Kaminski et al. 1991). However, the mutant strains retained partial nitrogenase activity (ca. 10 %) suggesting the existence of another regulatory circuit. Mutants of ORF140 are Nif$^+$, Fix$^+$ (Mandon et al. 1993).

Transcription of *nifA* is abolished by increased oxygen tension. *fixK*- and *nifA-lacZ* fusions were constructed and their properties indicated that *fixLJK* were involved in the oxygen control of *nifA* expression (Kaminski, Elmerich 1991; Kaminski et al. 1991). This oxygen regulatory pathway exhibits features common with those previously observed in *R. meliloti* (David et al. 1988; Batut et al. 1989). In both organisms, the two component regulatory proteins FixL and FixJ promote *fixK* transcription under oxygen limiting conditions. However, the Fnr-like protein FixK in *A. caulinodans* positively controls *nifA* and the *fixNOQP* operon (Figure 2), whereas in *R. meliloti* it is a negative regulator of *nifA* and a positive regulator of *fixN* (Batut et al.1989; Kaminski et al. 1991; Mandon et al..1994). This reflects the difference in the promoter structures of the *nifA* genes in the two bacteria. Indeed, the *A.caulinodans nifA* promoter region carries a motif resembling an anaerobox (Ratet et al. 1989; Nees et al.1988). This motif is also present upstream from *fixN* and ORF140 (Mandon et al. 1993,1994). Transcription starts of *fixN* and *nifA* were mapped by primer extension to 33 and 34 nucleotides respectively downstream from the anaerobox for each gene (Mandon et al. 1994, Kaminski unpublished). No signal was obtained with RNA preparation from a FixK mutant strain.

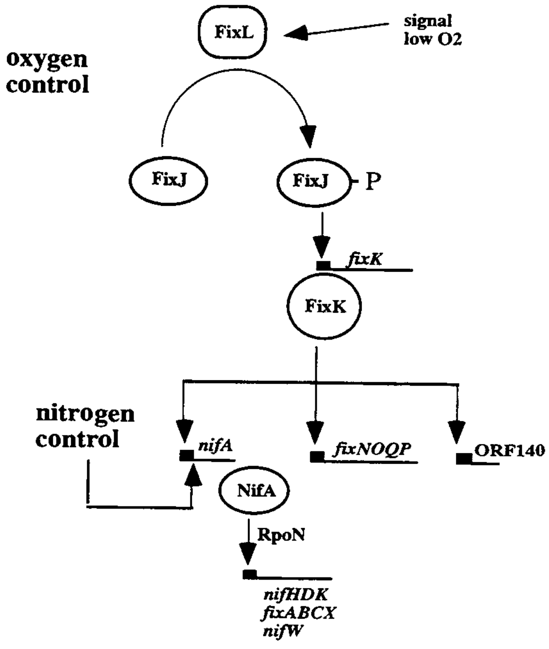

Figure2: Oxygen control of nitrogen fixation in *A. caulinodans* ORS571

Control by nitrogen: involvement of a regulator of the LysR family.

Several regulatory mutants impaired in the nitrogen control of nitrogen fixation have been described. Two pairs of two component regulatory systems, NtrBC and NtrYX, have been identified (Pawlowski et al. 1987, 1991). Strains carrying mutations of these genes fix nitrogen weakly both in the free-living, symbiotic states. NtrY carries two transmembrane domains. It was therefore proposed that NtrB could sense the intracellular nitrogen status and NtrY may respond to the extracellular nitrogen concentration. *nifA* expression is only partially affected in an *ntrC* or *ntrX* mutated background. In addition, *nifA* expression is independent of *rpoN* although the *nifA* promoter contains a typical -12/-24 consensus sequence. These observations led to the proposal that there is an alternative sigma factor specific for the *nifA* promoter (Stigter et al., 1993).

A pleiotropic negative mutant strain, 5795 was characterized (Denefle et al. 1987). This led to the identification of a new regulatory gene that controls nitrogen metabolism in *A. caulinodans* (Michel-Reydellet et al., this volume). Strain 5795 is a glutamine auxotroph, also impaired in the utilisation of amino acids as nitrogen sources, in nitrogen fixation. However *nifA* is expressed whereas the expression of *nifH* is totally abolished. A DNA fragment was isolated from the wild type strain which restored prototrophic growth and nitrogen fixation to strain 5795. The nucleotide sequence of a DNA fragment of 2.5 kb containing the complementing region was established. It contained an ORF whose translation product is very similar to transcription regulators of the LysR family, including Nac. Further characterisation of the *nac*-like gene is in progress.

Control of *nifA* transcription, translation: involvement of NrfA, an homologue of the *E. coli* RNA-binding protein HF-I.

Another Nif$^-$/Fix$^-$ regulatory mutant, strain 5751 (Denefle et al., 1987), unable to synthesise the polypeptides normally induced under conditions of nitrogen fixation in the wild type was studied. In contrast to strain 5795, 5751 is a prototroph. Using *lacZ* fusions it was shown that *nifA* and *nifA*-regulated genes were not expressed in this mutant. The mutation was complemented both by a constitutively expressed *nifA* gene or by a 1.1 kb DNA fragment from the wild type strain. The fragment contains a single ORF (*nrfA*) of 255 bp coding for a 85 amino acid polypeptide (Kaminski et al. 1994). The deduced amino acid sequence of this ORF (named *nrfA*) is similar to HF-I, a RNA binding protein in *E. coli*, which is required for replication of bacteriophage Qβ RNA. Southern blot analysis indicated that homologues of *nrfA* are present in the genome of other diazotrophs including rhizobia, *Klebsiella pneumoniae*.

A null mutant of *nrfA*, constructed by gene disruption, had the same phenotype as the point mutant (Kaminski et al.. 1994). Both the *E. coli hfq*, the *B. japonicum nrfA*-like genes restored nitrogen fixation to the *A. caulinodans nrfA* point, null mutant strains, suggesting that they encode functional homologues. However, *hfq* control has multiple targets in *E. coli*: disruption of *hfq* gene caused a broad pleiotropic phenotype (Tsui et al.. 1994). A *K. pneumoniae* mutant strain (Kaminski unpublished data), was also impaired for growth properties, but retained a specific nitrogenase activity of the same order of magnitude as the wild type. Consequently, the involvement of *nrfA* in the control of *nifA* expression reflects specific properties of *A. caulinodans*.

Experiments are in progress to test the hypothesis that NrfA controls the translation of *nifA*. In this model, the main transcriptional activator for *nifA* expression is FixK, which prevents *nifA* transcription at non permissive oxygen concentrations. None of NtrC, NtrX, "Nac", nor RpoN play a major regulatory role in *nifA* expression. The level at which the nitrogen controls nitrogen fixation is thus unclear.

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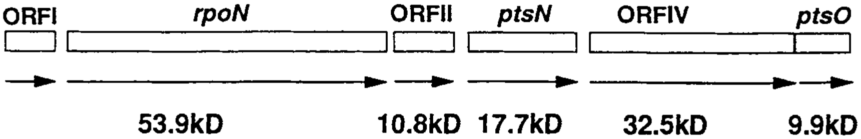
THE ROLE OF GENES DOWNSTREAM OF THE σ^N STRUCTURAL GENE *RPON* IN *KLEBSIELLA PNEUMONIAE.*

MERRICK M.J.[1], TAYLOR, M[1]., SAIER, M.H. Jr.[2] and REIZER, J.[2]
[1] Nitrogen Fixation Laboratory, University of Sussex, Brighton BN1 9RQ, UK.
[2] Dept. of Biology, University of California at San Diego, La Jolla, California, 92093-0116, USA

Introduction

The RNA polymerase sigma factor σ^N is unique amongst bacterial RNA polymerase sigma factors both in terms of its structure and in the mechanism of transcription initiation mediated by the $E\sigma^N$ holoenzyme (Kustu *et al.*, 1989; Merrick,1993). σ^N is widely distributed amongst the eubacteria and is required for transcription of a wide variety of genes, including for example flagellar genes in *Caulobacter*, xylene degradation genes in *Pseudomonas*, hydrogenase genes in *Alcaligenes* and nitrogen fixation genes in many, though not all, diazotrophs. The σ^N structural gene, *rpoN*, has now been cloned and characterised from at least 15 genera and in the majority of cases where sequence analysis has extended beyond *rpoN* a conserved set of linked genes has been identified (Merrick and Coppard, 1989; Merrick, 1993).

Fig.1 The *rpoN* operon in *E.coli* and *K. pneumoniae*



A conserved open reading frame (ORFI) is often found upstream of *rpoN*. This ORF encodes a protein which belongs to a family of ATP-binding proteins including NodI, HisP and MalK (Higgins *et al.*, 1986) but despite its linkage to *rpoN* there is presently no evidence that it is cotranscribed with *rpoN* and its function is unknown (Albright *et al.*, 1989).

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Although the genes downstream of *rpoN* are conserved in many organisms, their full extent has so far only been analysed in *E.coli* where a total of four genes have been described which together with *rpoN* appear to comprise a single operon (Jones *et al.*, 1994; Powell *et al.*,1995). The first gene (*orfII*) encodes a protein with marked homology to the products of a diverse group of genes that include a *pheA*-linked ORF in *E.coli* (Hudson and Davidson, 1984), an ORF downstream of *fliT* in *Bacillus subtilis* (Chen and Helmann, 1994), a light-repressed gene (*lrtA*) in *Synechococcus* (Tan *et al.*, 1994), and a gene encoding a ribosomally-associated protein in spinach chloroplasts (Johnson *et al.*, 1990).

The second gene has been designated *ptsN* owing to the marked similarity between its product and many phosphotransferase enzyme IIA proteins (Reizer *et al.*, 1992; Saier and Reizer, 1994). The third gene (*orfIV*) encodes a protein with limited similarity to a number of ATP-binding proteins and the final gene in the operon (*orfV*), which has so far only been reported in *E.coli*, has been designated *ptsO* in recognition of the marked homology between its product and the bacterial phosphotransferase protein HPr (Jones *et al.*, 1994; Saier and Reizer, 1994).

The phosphotransferase system (PTS) mediates the uptake and phosphorylation of many carbohydrates in bacteria (Postma *et al.*, 1993; Saier and Reizer, 1994) via a series of phosphoryl transfer proteins that catalyse the transfer of phosphate from phosphoenolpyruvate (PEP) to the transported sugar. Two proteins, Enzyme I and HPr, transfer phosphate from PEP to a sugar-specific Enzyme II complex that is localised to the inner membrane. The Enzyme II complex comprises three (or four) proteins or domains designated IIA, IIB, IIC (and sometimes IID) (Saier and Reizer, 1992). Enzyme IIA of the *E.coli* glucose-specific PTS (EIIA^{Glc}) is also thought to act as a regulator of the activity of a number of enzymes and transport proteins (see Postma *et al.*, 1993)

The homology of two of the gene products, PtsN and PtsO, encoded by genes in the *rpoN* operon, to proteins of the phosphotransferase system has led to the suggestion that a function of the proteins could be to coordinate carbon and nitrogen metabolism in the cell (Reizer *et al.*, 1992; Powell *et al.*, 1995). This suggestion is based on the σ^N-dependent transcription of many nitrogen-regulated genes and our earlier proposal that the products of *orfII* and *ptsN* might regulate Eσ^N activity (Merrick and Coppard, 1989). We have now sequenced the whole *rpoN* operon in *K. pneumoniae*, confirmed its homology to that of *E.coli* and examined the phenotypes of strains carrying mutations in *orfII*, *ptsN* and *ptsO*. We have also studied PtsN phosphorylation *in vitro* and in the light of our results we suggest that the *rpoN*-linked genes may encode a sensory system that monitors the energy status of the cell and modulates σ^N-dependent transcription accordingly.

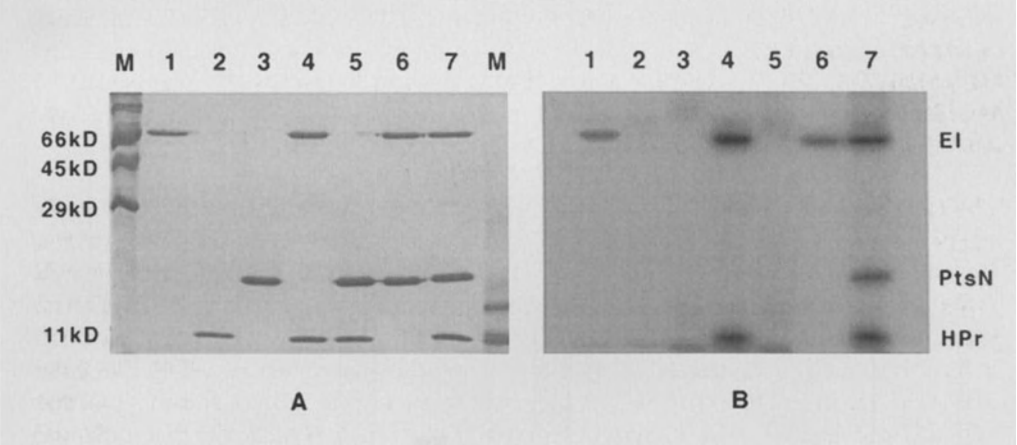
Sequence of the *K. pneumoniae rpoN* operon

We have now sequenced a total of 2.1kb downstream of *rpoN* in *K. pneumoniae*. This extends our original sequence (Merrick and Coppard, 1989) by 0.6kb and confirms the presence of *orfIV* and *ptsO*. The predicted translation products of *orfIV* and *ptsO* are almost identical to those of the equivalent genes in *E.coli* (Jones *et al.*, 1994) and the sequence suggests that *rpoN*, *orfII*, *ptsN*, *orfIV* and *ptsO* constitute a single operon.

Phosphorylation of PtsN

The observed homology between the *ptsN* product and phosphotransferase enzyme IIA proteins suggests that this protein might be capable of being covalently modified by phosphorylation in a phosphoenolpyruvate (PEP) - dependent manner. Furthermore such phosphorylation could potentially be mediated by the phosphotransferase proteins Enzyme I (EI) and HPr. To test this hypothesis we carried out *in vitro* phosphorylation experiments with purified proteins and showed that PtsN can be phosphorylated in the presence of [^{32}P]-PEP in a manner that is dependent upon the presence of both EI and HPr (Fig. 2).

Figure 2. *In vitro* phosphorylation of PtsN.



A. Coomassie-stained SDS PAGE of proteins after 10 min incubation with [^{32}P]-PEP.
M - molecular weight markers

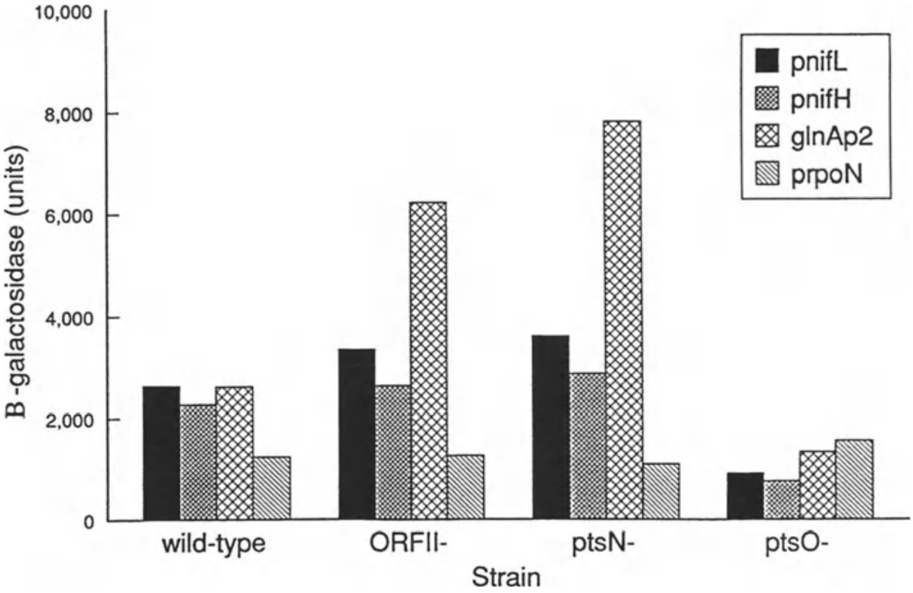
B. Autoradiograph of gel shown in A identifying proteins phosphorylated in each reaction.

Effects of mutations in *orfII*, *ptsN* and *ptsO* on transcription from Eσ^N-dependent promoters

In our earlier studies (Merrick and Coppard, 1989) we reported the effects of an *orfII::kan* mutation on expression from certain σ^N-dependent promoters. We have now studied expression from plasmid-borne translational *lacZ* fusions to the promoters of *rpoN*, *nifL*, *nifH*, and *glnA*p2 in strains carrying *orfII::kan*, *ptsN::kan* and *ptsO::kan* mutations.

Expression from p*rpoN* is unaffected by any of the mutations, indicating that the *rpoN*-linked genes do not regulate transcription of *rpoN*. Mutations in *orfII* and *ptsN* cause elevated expression from p*nifL*, p*nifH* and *glnA*p2. This elevation is most apparent at intermediate levels of fixed nitrogen, i.e under conditions where the promoters are not fully derepressed, and is most pronounced with *ptsN::kan* (Fig.2). By contrast a *ptsO::kan* mutation has the reverse effect with a significant decrease in expression from the same promoters. In all cases the effects are seen most clearly with *glnA*p2 (Fig. 2). The effects seen using promoter fusions were also reflected in measurements of nitrogenase activity (using acetylene reduction assays) where the phenotypes of the mutations were even more marked (data not shown).

Figure 3. Effects of mutations in the *rpoN*-linked genes on expression from various promoters. (cells grown in limiting nitrogen - 200µg/ml ammonium sulphate)



Conclusions

We have established that the five genes that are proposed to constitute the *rpoN* operon in *E.coli* are fully conserved in *K. pneumoniae*. *OrfIV* encodes a protein with 98% identity in primary amino acid sequence to the analogous *E.coli* gene and the *orfV* (*ptsO*) sequences encode proteins with 91% identity in the two organisms (Jones *et al.*, 1994). It is also of interest to note that *orfIV* and *pstO* are translationally coupled in both organisms implying that there may be a functional requirement for synthesis of equimolar amounts of both polypeptides.

What is the role of the *rpoN*-linked genes?

The conservation of genes both upstream and downstream of *rpoN* is now established as a common feature in many bacterial genera. The extent of conservation of the other genes (*ptsN*, *orfIV* and *ptsO*) remains to be determined but in most cases where sequencing has been extended downstream of ORFII, homologous genes have been found and hence it is tempting to speculate that these genes play a role in the function of σ^N.

We have shown that PtsN can be phosphorylated in an EI, HPr-dependent manner *in vitro* and it seems likely that this could also occur *in vivo*. However as the *in vitro* phosphorylation experiments are not carried out under physiological conditions the precise nature of the phosphotransfer reactions that occur *in vivo* cannot yet be described. Nevertheless the reciprocal phenotypic effects of mutations in *ptsN* and *ptsO* are consistent with phosphotransfer between the products of these two genes (Powell *et al.*, 1995) such that a mutation in one of the genes prevents phosphorylation of the product of the other gene and locks it in a single conformation. As PtsN can exist in both a phosphorylated and non-phosphorylated form it has the potential to function like $EIIA^{Glc}$ as an indicator of a certain cellular metabolic state. However previous suggestions that the function of the system is to coordinate carbon and nitrogen metabolism do not address the fact that not all σ^N-dependent genes are involved in nitrogen metabolism e.g *E.coli* hydrogenase genes or xylene degradation genes in *Pseudomonas*.

The phenotypic effects of mutations in *orfII*, *ptsN* and *ptsO* which we have so far characterised are all manifested in alterations in expression from σ^N-dependent promoters. Expression from other promoters such as p*rpoN* or *gal*p2 is not affected by these mutations (Merrick and Coppard, 1989). Transcription initiation from σ^N-dependent promoters occurs by a common mechanism (Kustu *et al.*, 1989) requiring a particular class of activator protein. In all known cases RNA polymerase containing σ^N is capable of forming a relatively stable closed complex with the promoter but only undergoes the transition to an open complex in the presence of the requisite activator (Popham *et al.*, 1989). Furthermore transcription initiation requires ATP hydrolysis by the activator protein (Weiss *et al.*, 1991; Austin and Dixon, 1992). Hence σ^N-dependent transcription is characterised by a requirement for ATP and it is therefore possible that in the event of an energy limitation it might be advantagous for σ^N-dependent transcription to be down-regulated.

A number of studies have indicated a link between the energy state of the cell and PTS carbohydrate uptake catalysed by Enzyme IIs (Reider *et al.*, 1979; Postma *et al.* 1993). Hence it is possible that the state of phosphorylation of PtsN could reflect the cellular energy state. We therefore suggest that the role of the *rpoN*-linked genes might be to integrate expression from σ^N-dependent promoters with the cellular energy status. This could be achieved by using the phosphorylation state of PtsN to communicate directly or indirectly with the transcription apparatus. In this case the target of this regulatory system could be either the σ^N-containing holoenzyme or the activator proteins all of which possess common structural and functional characteristics. Elaboration of this model requires further analysis, both genetic and biochemical, of the *rpoN*-linked genes and their products.

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USE OF THE LUCIFERASE (TN5-*LUX*) REPORTER SYSTEM TO STUDY *RHIZOBIUM MELILOTI* GENES RESPONDING TO N/C/O2 LIMITATION OR PLANT FACTORS AND THEIR ROLE IN RHIZOSPHERE COMPETITION.

DE BRUIJN F.J.[1,2,3], **GRAHAM L.**,[1] **MILCAMPS A.**[1,3], and
RAGATZ D.[1,3] MSU-DOE Plant Research Laboratory[1], Department of
Microbiology[2], and NSF Center for Microbial Ecology[3], Michigan State
University, E. Lansing, MI 48824 USA.

The induction of nitrogen-fixing root and stem nodules on leguminous plants by soil bacteria belonging to the Rhizobiaceae is a highly evolved, complex process requiring a fine-tuned interaction between the bacteria and their host. Recently, considerable progress has been made in elucidating the signal exchange between rhizobia and their host plant, leading up to the activation of the microbial nodulation (*nod/nol*) genes, Nod factor biosynthesis and subsequent Nod factor mediated initiation of nodule ontogeny. Moreover, the molecular basis of rhizobial nitrogen fixation (*nif/fix*) gene induction in response to physiological cues such as oxygen (and nitrogen) availability has been studied in detail. However, much less is known about the influence of the persistence and competitive ability of rhizobia in the soil and the rhizosphere of plants on the very early stages of rhizobial infection. This is particularly relevant when one is interested in developing improved strains of nitrogen fixing rhizobia, since they tend not to be competitive when reintroduced into the soil or rhizosphere of plants (see Dowling, Broughton, 1986).

A complex set of biotic signals, including nutritional factors and plant-secreted signal molecules, is responsible for structuring microbial communities in the rhizosphere, and this, in turn, influences the rhizobial infection process. To increase our understanding of microbial colonization of the rhizosphere of plants and its relevance to symbiotic nitrogen fixation, we wish to understand the molecular basis of the rhizobial response to common environmental stresses and to plant factors secreted into the rhizosphere, to develop user friendly methods for the detection and classification of soil microbes, and to explore the use of specific nutritional mediators to create "biased rhizospheres" (Lim et al., 1993; Phillips et al., this volume; de Bruijn, 1992; Versalovic et al., 1994; Rossbach et al., 1994).

Rhizobial response to environmental (nutritional) stresses.

Soil bacteria in the environment are frequently exposed to nutrient starvation conditions. In fact the majority of non-rhizosphere soil is so oligotrophic it has been called a "nutritional desert" (Metting, 1985). Consequently, bacterial growth is often extremely limited and non-growth, or very low growth, may be considered as the norm (Kjelleberg et al., 1987). The mean generation times of bacteria in the soil have been estimated to be from less than 1 to 80 per year (Shields et al., 1973; Gray, 1976; Lynch, 1988; Matin, 1991) for bacteria whose generation times occur within

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hours in a lab setting. Thus, understanding how bacteria survive and persist under these conditions is important both in terms of increasing our fundamental knowledge of the microbial ecology of the rhizosphere, as well as in applied contexts such as the release of genetically improved rhizobia or other modified microorganisms (GEMs) into the environment.

Most of what is known about starvation responses in non-differentiating bacteria comes from studies on the induction of proteins by carbon, phosphorus, or nitrogen starvation in *Echerichia coli, Salmonella typhimurium,* and *Vibrio* spp., using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (Matin, 1991; Spector, Cubitt, 1992; Nyström et al., 1990). Many of these polypeptides are uniquely induced by starvation for a single nutrient. However, several of them are induced by two starvation conditions, and a substantial subset is induced under all starvation conditions examined.

Although much is known about bacterial systems in the laboratory, where bacteria are grown under ideal conditions, comparatively little is understood about their behaviour under unfavorable conditions, such as in the soil. Moreover, little work has been carried out with common soil isolates, which may have different strategies for dealing with nutrient starvation than enteric or marine bacteria. Recently, a large number of proteins induced by carbon and nitrogen starvation were found in *Pseudomonas putida* (Givskov et al., 1994). As in the case of *E. coli* and *Vibrio* spp., these proteins are temporally regulated, and include a large group of eight proteins induced by multiple stresses.

To date, few roles have been assigned to the large collection of induced proteins in any of the bacterial species examined and little is known about how corresponding mutations in the loci coding for these proteins affect survival and competition in the natural habitat. Therefore, we have taken a different approach for investigating starvation-induced loci in soil bacteria, by mutagenizing the genome of *R. meliloti* with a Tn*5* derivative (Tn*5*-1063), containing promoterless *luxAB* genes (Wolk et al., 1991) encoding luciferase. This transposon creates transcriptional fusions, bringing the *luxAB* genes under the control of the tagged gene promoter. Under appropriate conditions, these gene fusions will express luciferase, resulting in bacterial bioluminescence (Meighen, Dunlap, 1993).

The use of Tn*5* has been shown to be an extremely useful tool for random mutagenesis in many Gram-negative bacterial systems including *R. meliloti* (see de Bruijn, Rossbach, 1994). In addition, the use of luciferase genes such as *lux* and *luc* as reporters has proven to be useful to mark strains and in studies of bacterial gene expression in genera in the soil and rhizosphere (see Meighen, 1991; Prosser, 1994; Cebolla et al., 1993; Moeller et al., 1994; Jansson, 1995)

The Tn*5*-*lux* system provides us with an easy method to isolate tagged genes of interest, as well as a sensitive assay for the study of induction of these genes. In addition, these Tn*5* mutant strains can be analyzed for their biochemical and physiological properties. Most importantly, these mutants can be used to conduct soil persistence and competition experiments in order to understand the role of the tagged genes in structuring the bacterial populations in the soil and rhizosphere. Recently, we used this approach successfully in *Pseudomonas fluorescens* to isolate and characterize gene fusions whose bioluminescence was induced under nitrogen and phosphate starvation (Kragelund et al., 1995).

1. Generation and characterization of 5000 *R. meliloti* Tn5--1063 insertion mutants.

Using the Tn*5*-1063 transposon, a collection of 5000 insertion mutants of *R. meliloti* 1021 was generated and stored in microtiter plates. The insertional specificity of the Tn*5*-1063 transposon in *R. meliloti* was examined by determining both the frequency and distribution of auxotrophs, and by Southern blot analysis. The frequency of auxotroph generation was 1.2%, and multiple auxotrophic phenotypes were identified. Southern blotting analysis of these auxotrophs revealed different sizes of hybridizing bands for each isolate. Thus, both analyses suggest that the transposition frequency of Tn*5*-1063 in *R. meliloti* is high and its insertional specificity low, making it a useful tool for our studies.

2. Identification and characterization of *R. meliloti* genes induced by N-, C-, or O_2 limitation.

In order to identify genes induced by nutrient limitation/stress, filters carrying the Tn*5*-1063 insertion mutant strains were transferred from rich medium (aerobic conditions) to the appropriate stress media and screened for colonies with significantly higher light emission under stress versus non-stress conditions, using the Hamamatsu photonic camera.

Twenty two different N-starvation, 12 C-starvation and 32 oxygen limitation induced *R. meliloti* loci were isolated. The Tn*5*-1063 tagged gene segments were cloned from 31 of the strains carrying the induced gene fusions and partially characterized by DNA-sequencing (Lim et al., 1993; D. Ragatz, P.O. Lim, L. Kragelund and F.J. de Bruijn, unpublished results). Some of the Tn*5*-1063 tagged loci were found to correspond to already characterized microbial genes, while others represent novel loci. For example, N- starvation induced loci included nitrate and nitrate- reductase (*nasAB*), and exopolysaccharide (*exoYF*) biosynthesis genes, C-starvation induced loci included putidaredoxin-reductase (*bedA*), ribose transport protein (*rbsA*) and methanol dehydrogenase (oxidation; *moxF*) genes and O_2 limitation induced loci included dehydrogenase (*acoD*) and aminoacid transport-like genes (*braF*), exopolysaccharide biosynthesis (*exoO*), cytochrome oxidase (*cyoC*) and nitrogen fixation (*fixN*) genes.

Table 1. Characterization of Tn5-1063 insertion mutants of *R. meliloti*

Induction by:	# Isolated:	Similarity to Known Genes:
Nitrogen-starvation	22	*nas*AB, *exo*FY
Carbon-starvation	13	*bed*A , *rbs*A , *mox*F
Low oxygen tension	34	*aco*D, *fix*N, *bra*F, *exo*O, *cyo*C

Surprisingly, in contrast to the results obtained in enteric bacteria and marine organisms (see above), only one Tn*5*- 1063 tagged locus of *R. meliloti* was found to be induced by both C- and N- stress and one by both oxygen and C-limitation. The genes tagged in these two strains did not share homology with any previously identified genes/proteins in the databases, suggesting that they represent novel loci which may be involved in a more general response to low growth-rate/starvation conditions.

3. Role of Tn*5*-1063 tagged, stress-induced loci of *R. meliloti* in soil persistence and competition.

Studies were initiated to examine the importance of these genes in competition and survival. Several strains carrying fusions induced by nitrogen- and carbon-starvation were inoculated in sterile nutrient-rich soil and examined for their ability to survive compared to the wild-type under the same conditions, by plate counts over the course of eight weeks. Several independent experiments showed that survival of the mutants under these conditions was not significantly different from that of the wild-type. Two of the carbon strains were also examined for their competitive (growth) ability in soil. The mutants and the wild-type were coinoculated into sterile soil at a 1:1 ratio. Both a nutrient-rich soil, and a nutrient-poor soil were inoculated with the same cultures. Plate counts were carried out at two-week intervals, in order to compare survival rate. The results showed a greater than 100-fold decrease in numbers of mutant bacteria compared to the wild-type and indicate that the wild-type can out-compete the mutants in many cases, particularly in nutrient-poor soil.

The carbon and some nitrogen mutants were also examined for their ability to competitively nodulate in the presence of the wild-type strain. Cultures of mutants and wild-type were inoculated on one-week old alfalfa roots at a 10:1 mutant:wild-type ratio. After seven weeks, nodules were collected and surface-sterilized, crushed in sterile microcentrifuge tubes and the bacteria plated on selective media, in order to determine the percentage of each strain present in the nodules. Many of the mutants were completely, or significantly, absent from nodules, even though they were able to produce effective nodules when inoculated on alfalfa roots by themselves (except for the two exopolysaccharide mutants, N112 and N149). This indicates that many of the genes induced by carbon and nitrogen starvation play a role in competition for nodulation.

4. Regulation of nitrogen starvation-induced Tn*5*-1063 tagged *R. meliloti* loci.

A study was initiated to reveal the regulatory pathways of the nitrogen starvation induced genes in *R. meliloti*. For this purpose, the gene fusions were cloned into broad host range vector pLAFR1 and transferred by conjugation into *R. meliloti* wild type, and mutant strains altered in their regulation of general nitrogen assimilation (*ntrA* and *ntrC* mutants). Measurement of a unchanged *lux* expression in the wild type strain and the genetic backgrounds showed that the majority of the gene fusions were not regulated by the *ntr* system, revealing the involvemant of more than one regulator. Four gene fusions, however, were shown to be *ntr* controlled for their *lux* expression, among which are the *nasA* and *nasB* loci, encoding assimilatory nitrate and nitrite reductase. At present, secondary mutagenesis is being carried out with some of the nitrogen starvation induced mutants in order to isolate the novel (non-*ntr* system) regulators for the nitrogen starvation induced loci.

Rhizobial response to plant-derived factors.

In order to understand how plants may be involved in affecting rhizobial populations in the rhizosphere, we also used our collection of 5000 Tn*5*-1063 insertion mutants to identify *R. meliloti* genes induced by factors secreted or exuded by plant roots and seeds, in collaboration with Dr. Don Phillips in Davis, CA (see Phillips et al., this volume for a detailed discussion). We have postulated that specific compounds such as flavones and betaines may be involved in the structuring of microbial communities in the rhizosphere of plants (including rhizobial populations) and therefore have started a search for genes induced by such factors (which are not already identified nodulation genes) using the same Tn*5*-*lux* screen that has been employed to identify nutrient limitation induced genes.

We screened the collection of 5000 Tn*5* -*luxAB R. meliloti* mutants for induction of bioluminescence by a cocktail of luteolin, DHF, quercetin, formononetin, stachydrine, and trigonelline, each at 10μM. Responsive fusions were identified as those which increased in luminescence after four hours from transfer from GTS medium to GTS + cocktail. We have identified 23 responsive fusions. We have discovered that these responses are difficult to discern unless the mutants are simultaneously starved for nitrogen.

Initial experiments to determine which fusions responded best to a particular inducer have given mixed results from plate cultures; however, stachydrine appears to be an unexpectedly strong inducer relative to the flavonoids, which typically induce at 1000-fold lower concentrations than the betaines. It may be that, in our screening system, the water-soluble betaines have more access to (entry into) the rhizobial colonies than do the hydrophobic flavonoids. Of 16 mutants tested, 12 are Nod+Fix+, and 4 are Nod-Fix- (may carry insertions in already known *nod* /*nol* genes) and none appeared to be affected in motility (see Table 2).

Mutant	Nodulation	N-fixation	Motility
4L7	-	-	+
5B4	+	+	+
11D7	-	-	+
12J3	-	-	+
13E4	-	-	+
31C5	+	+	+
31D2	+	+	+

Table 2. Nodulation, nitrogen fixation, and motility characteristics of selected mutants.

All mutants contain single Tn*5* insertions as seen by Southern hybridization and initial sequencing efforts indicate that in one mutant, the transposon is located in an ORF with no sequence similarity to protein sequences in the GenBank/EMBL databases, while in another it is located within a gene for a protein with similarity to a potato mitochondrial processing peptidase. This gene shows no similarity to known bacterial peptidases or proteins with export functions.

In rich soil, none of the mutants tested appeared to be affected in persistence, relative to the wild-type strain. Experiments to test their persistence in poor soil and their competitive ability in nodule occupancy experiments are in progress. In addition, the chemotactic behaviour of selected mutant strains towards flavones or betaines is being studied.

Conclusions.

In this paper, we have described our experimental approach to isolate and characterize *R. meliloti* genes involved in persistence and competition in the soil and rhizosphere of plants. By identifying genes induced by environmental stresses and plant factors using a Tn*5*-*lux* reporter gene transposon, we also hope to gain new insights into the environmental factors controlling early events in rhizobial infection of plants and into ways that plants may structure microbial communities in the rhizosphere. These insights may prove to be important for more efficient introduction of superior nitrogen fixing strains into agricultural systems.

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REGULATION OF MOLYBDENUM AND ALTERNATIVE NITROGENASES IN THE PHOTOSYNTHETIC PURPLE BACTERIUM *RHODOBACTER CAPSULATUS*

KLIPP W., ANGERMÜLLER S., ASTROTH S., KAMP P.-B.,
KERN M., KUTSCHE M., LEIMKÜHLER S., PASCHEN A.
Lehrstuhl für Genetik, Universität Bielefeld, Postfach 100131,
D-33501 Bielefeld, Germany

The photosynthetic nonsulfur purple bacterium *Rhodobacter capsulatus* is able to fix dinitrogen (N_2) using either a conventional molybdenum nitrogenase or an alternative, heterometal-free nitrogenase. Until now, thirtyfive genes have been characterized in detail that are involved in synthesis and regulation of the molybdenum nitrogenase (Klipp, 1990; Schmehl et al., 1993). These genes are localized in three distinct regions of the *R. capsulatus* chromosome, designated *nif* regions A, B and C. Expression of *nif* operons is controlled by a regulatory circuit similar to the Ntr-system of enteric bacteria (Fig.1). *R. capsulatus nifR1*, *nifR2* and *nifR4* are homologous to *ntrC*, *ntrB* and *rpoN*, respectively. Under conditions of nitrogen limitation this *nif* specific Ntr-system activates two functional copies of *nifA*, which in turn activate all other nitrogen fixation genes. However, in contrast to the Ntr system of enteric bacteria, no genes involved in the general nitrogen metabolism are activated by *R. capsulatus* NifR1. Mapping of transcriptional startpoints of $nifA_I$ and $nifA_{II}$ revealed a proximal -23/-7 promoter element (Foster-Hartnett, Kranz, 1992; Preker et al., 1992) which might be the recognition site of an RNA polymerase that contains an as yet unidentified sigma factor (r^X in Fig.1). Transcriptional activity of this polymerase strictly depends on binding of the phosphorylated form of NtrC to tandemly arranged distal DNA elements (Foster-Hartnett et al., 1994). The gene products of $nifA_I$ and $nifA_{II}$ belong to the group of oxygen-sensitive NifA proteins and act as transcriptional activators for the expression of all other *nif*

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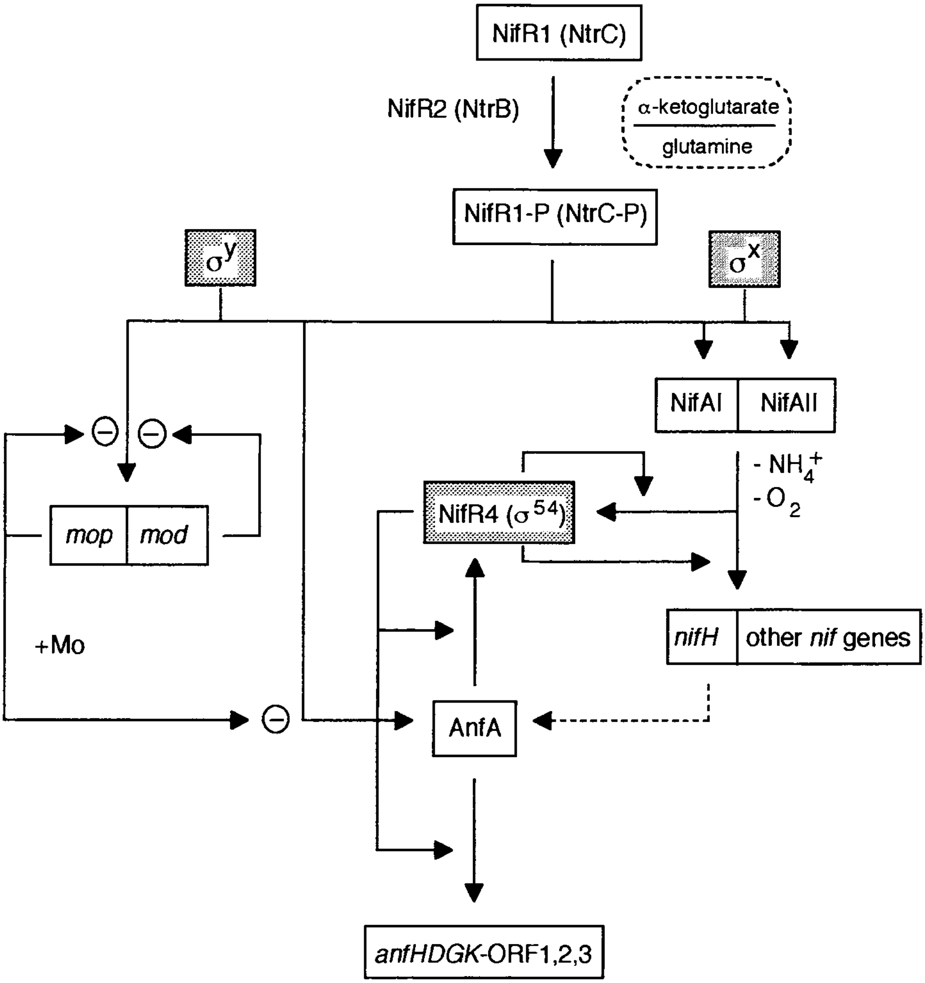
NifR1 (NtrC)
NifR2 (NtrB)
α-ketoglutarate
glutamine
NifR1-P (NtrC-P)
σ^y
σ^x
NifAI
NifAII
⊖
⊖
mop
mod
- NH4+
-O2
NifR4 (σ54)
+Mo
nifH
other nif genes
⊖
AnfA
anfHDGK-ORF1,2,3

Fig.1: Regulatory cascade of *R. capsulatus* that controls genes encoding the molybdenum nitrogenase, the alternative nitrogenase and a high affinity molybdenum transport system (for details see text).

genes together with RNA polymerase containing σ^{54}. This sigma factor is encoded by *nifR4* and transcriptionally controlled by an autoregulatory circuit consisting of NifA and NifR4 itself (Cullen et al., 1994).

In addition to the molybdenum nitrogenase, *R. capsulatus* harbors a genetically distinct alternative nitrogenase, which corresponds in all aspects to the iron-only nitrogenase of *Azotobacter vinelandii* (Schneider et al., 1991). The synthesis of an active alternative nitrogenase is not only dependent on a set of specific *anf* genes but also on genes required for the molybdenum nitrogenase (Schüddekopf et al., 1993). These genes comprise *nifB* and *nifV* encoding proteins involved in cofactor syntheses and *rnfACDEF* and *fdxN*, which code for components of a putative membrane complex necessary for electron transfer to nitrogenase (Schmehl et al., 1993). In addition, the regulatory genes *nifR1* (NtrC), *nifR2* (NtrB) and *nifR4* (σ^{54}) are absolutely required for alternative nitrogen fixation. The analysis of a large number of Tn*5* mutants, unable to fix nitrogen via the alternative nitrogenase, indicated that all genes specific for the alternative nitrogenase are clustered in the chromosome. The complete nucleotide sequence of this *anf* gene region revealed *anfA*, coding for a transcriptional activator specific for alternative nitrogen fixation, the structural genes of the alternative nitrogenase (*anfHDGK*) and three open reading frames (ORF1, ORF2 and ORF3). ORF1 and ORF2 are homologous to two open reading frames located at a corresponding position downstream of *A. vinelandii anfHDGK* (Joerger et al., 1989). In contrast, no homologue to ORF3 could be identified in *A. vinelandii* and no proteins exhibiting significant homology to ORF3 are present in data bases. Genetic analysis demonstrated that ORF1-3 are cotranscribed with *anfHDGK* and are essential for alternative nitrogen fixation. Although the capacity to grow with dinitrogen is severely impaired in ORF1-3 mutants, acetylene is reduced to normal amounts of ethylene and ethane.

Analysis of the promoter elements in front of the *anfHDGK*-ORF1,2,3 operon revealed a putative -24/-12 promotor, a sequence homologous to the binding site of the integration host factor (IHF) and two stretches of DNA resembling the binding site of AnfA from *A. vinelandii* (Austin, Lambert, 1994). The analysis of *R. capsulatus* mutants defective in σ^{54} (*nifR4*) and primer extension mapping proved the σ^{54}-dependent promoter in front of *anfH*. The analysis of fusions between various *anfH* upstream deletions and *E.coli lacZ* in *R. capsulatus* wild-type and in an IHF mutant background demonstrated that *anfH* expression strongly depends on the presence of the putative AnfA binding site. Furthermore, for full activity of the *anfH* promoter IHF is necessary as well.

The expression of *anfA* encoding the transcriptional activator of the *anfHDGK*-ORF1,2,3 operon was shown to be negatively regulated by ammonia and molybdenum. These regulatory properties are also characteristic for the expression of *mopA* and *modABCD*, which code for a molybdo-pterin-binding protein and a high affinity molybdenum transport system, respectively (Wang et al., 1993). As shown in Fig.1, the ammonia control of both, *anfA* and the *mopAmodABCD* operon, is also achieved by the NtrB/C homologues. Therefore, not only the molybdenum nitrogenase but also the alternative nitrogenase system as well as the molybdenum uptake system are part of the NtrC regulon in *R. capsulatus*. Although two tandemly arranged sequences, which correspond perfectly to the NtrC binding sites mapped in front of *glnB*, $nifA_I$ and $nifA_{II}$ (Foster-Hartnett, Kranz, 1994; Foster-Hartnett et al., 1994), are located upstream of *anfA* and *mopA*, respectively, no sequence resembling the putative recognition site of σ^x could be identified. Instead, primer extension mapping of the *anfA* and *mopA* transcripts revealed a novel type of promoter element located at position -45/-25. This result indicates, that in contrast to all NtrC-like proteins studied so far, *R. capsulatus* NtrC acts not together with σ^{54}-RNA polymerase but instead, at least two, yet unidentified sigma factors (σ^x and σ^y in Fig.1) are responsible for the NtrC-dependent ammonia control of the nitrogen fixation regulon.

The 5' termini of the NtrC-activated *anfA* and *mopA* transcripts are located within DNA regions of dyad symmetry. These inverted repeats share not only sequence homology to each other but also to DNA sequences in front of *A. vinelandii anfA* and *mopA*, two genes assumed to be regulated by molybdenum, and in front of the molydenum-repressed *modABCD* operon of

E. coli (Rech et al., 1995). To test whether these structures are target sites for negative regulation by molybdenum we constructed different deletion mutants. An *anfA* promoter mutant lacking the 3' part of the inverted repeat was no longer negatively regulated by molybdenum indicating that this DNA structure might be the target for a molybdenum regulated repressor.

Regulation of nitrogen fixation in *R. capsulatus* is not confined to the transcriptional level. Firstly, both nitrogenase systems are subjected to post-translational regulation that inactivates the nitrogenase reductases by ADP-ribosylation under conditions of either ammonia or oxygen supply or light limitation. The covalent modification of NifH and AnfH proteins is reversible and performed by the gene products of *draT* and *draG* (Masepohl et al., 1993). Secondly, the activity of the transcriptional regulator NifA is repressed by high NH_4^+ concentrations (Hübner et al., 1993). To analyse if this post-translational ammonia control is specific for *R. capsulatus* NifA or might be a general property of interdomain linker containing NifA proteins, we introduced constitutively expressed *nifA* genes from *Rhizobium meliloti* and *Klebsiella pneumoniae* into *R. capsulatus*. Although both heterologous *nifA* genes were able to complement an *R. capsulatus* $nifA_I/nifA_{II}$ double mutant, the activity of neither of these proteins was affected by ammonia. This result indicated that the ammonia-sensitivity is an intrinsic and specific property of *R. capsulatus* NifA. To identify factors that mediate ammonia dependent inactivation of NifA, a random Tn*5* mutagenesis was performed in an *R. capsulatus* strain carrying a *nifH-lacZ* fusion and a constitutively expressed $nifA_I$ gene. The analysis of mutants able to express *nifH* even in the precence of ammonia revealed that *hvrA* is involved in the post-translational regulation of NifA activity. This gene, located in a cluster of genes involved in regulation of photosynthesis, was previously shown to be responsible for low light activation of the photosynthetic apparatus (Buggy et al., 1994). Detailed analysis of an interposon induced *hvrA* mutant demonstrated that not only the ammonia control of NifA is altered in this mutant strain but also the oxygen regulation. This result indicated that *hvrA* might be involved in a more general control function linking the regulatory circuits of nitrogen fixation and photosynthesis.

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NITROGENASES AND HYDROGENASES IN CYANOBACTERIA

BOTHE H., SCHMITZ O., BOISON G., HUNDESHAGEN B.,
ZIMMER W.
Botanisches Institut, Universität zu Köln, Gyrhofstr. 15, D-50923 Köln,
Germany

A. Nitrogenases

Apart from the conventional, Mo-containing nitrogenase, the cyanobacterium *Anabaena variabilis* can express at least two alternative N_2-fixing enzyme complexes. This cyanobacterium grows with V in a Mo-deficient medium. The nitrogenase then expressed reduces C_2H_2 partly beyond C_2H_4 to C_2H_6 and produces more H_2 than the Mo-enzyme (Kentemich et al., 1988; Yakunin et al., 1991). Genes for this V-nitrogenase, *vnf*DGK (Thiel, 1993) and *nif*B (commonly used for both nitrogenases) (Lyons and Thiel, 1995) have subsequently been cloned, mapped and sequenced. It is generally agreed that *A. variabilis* contains a V-nitrogenase. In addition, there is physiological evidence that *A. variabilis* also contains an Fe-only nitrogenase (Kentemich et al., 1991). After an extensive state of N-deprivation where the cells totally bleached due to the utilization of phycobilins as N-reserve, *A. variabilis* restarted to grow slowly. The cultures reduced C_2H_2 partly to C_2H_6 and produced H_2 with a high rate. Determination by atomic absorption spectrometry showed that the concentration of Mo in the medium was <10 nM and of V <20 nM and likely too low for the expression of a Mo- or a V-nitrogenase. The cells showed an outburst in C_2H_6- and H_2-formations 3 h after the addition of Mo to the medium which typically happens with the Fe-only hydrogenase from *Azotobacter vinelandii* (Pau et al., 1989). Hybridizations of the *anf*H and *nif*H probes with genomic DNA from *A. variabilis* gave at least two distinct bands (Kentemich et al., 1991). Supporting evidence for the existence of an Fe-only nitrogenase came from Ni et al., (1990). The gene set coding for this enzyme complex has, however, not yet been found which leaves, of course, doubts about the meanings of these physiological results.

A new development in the field has recently been forwarded independently by two groups (Thiel, 1994; Schrautemeier, 1994; Schrautemeier et al., 1995). Concomitantly with a second copy of the *fdx*H gene (its product probably is the immediate electron carrier to nitrogenase), a second gene set, termed *nif*HDK2, could be detected in *A. variabilis*. These genes *fdx*H2 and *nif*HDK2 are transcribed exclusively under anaerobic conditions, early (< 4 h) after N-depletion in vegetative cells and apparently

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independently of heterocyst differentiation. With respect to its O_2-sensitivity, this alternative nitrogenase resembles the enzyme complex of the filamentous, non-heterocystous *Plectonema boryanum*. In *A. variabilis*, the expression of this alternative nitrogenase is apparently independent of a Mo-depletion in the medium, and the gene cluster apparently does not contain the G gene. Therefore this alternative nitrogenase is suggested to contain Mo in the active center, although it has not been characterized biochemically. Its discovery is somewhat surprising, since earlier experiments with *A. cylindrica* using nitrogenase antibodies (Murry et al., 1984) and with *Anabaena* PCC 7120 utilizing promoter$_{\mathit{nif}\mathrm{HDK}}$-*lux*AB reporter fusions (Elhai and Wolk, 1990) indicated the location of nitrogenase exclusively in heterocysts. With respect to its N_2-fixing capabilities, *A. variabilis* could well be totally different from other studied cyanobacteria (*A.* 7120, *Anabaena cylindrica*), since the V-nitrogenase also occurs only in *A. variabilis* (Kentemich et al., 1988) and presumably in the *Anabaena* sp. isolated from the fern *Azolla* (Thiel, 1993).

B. Hydrogenases

Despite the fact that isolated nitrogenases from all organisms produce H_2 with substantial rates, no or marginal net productions are generally observed in experiments with intact cyanobacteria. This is due to the fact that the H_2 evolved by nitrogenases is immediately recycled by hydrogenases. Cyanobacteria possess at least two different of such enzymes catalyzing the reaction $H_2 \Rightarrow 2\ H^+ + 2\ e^-$. One enzyme is called uptake hydrogenase, as it catalyzes only H_2-consumption with methylene blue (= MB) or phenazine methosulfate (= PMS) as electron acceptor, but virtually not the evolution of the gas. It is particularly (Tel-Or et al., 1978; Eisbrenner et al., 1981), if not exclusively (Peterson and Wolk, 1978), active in heterocysts of cyanobacteria. Independent evidence by Peschek (1979) and Eisbrenner et al. (1978), however, indicated that an uptake hydrogenase also occurs in the unicellular, non-N_2-fixing *Anacystis nidulans* (= *Synechococcus leopoliensis*). Currently it is not clear whether *Anabaena* and *Anacystis* contain two unrelated uptake hydrogenases, since these enzymes have not been characterized biochemically.

Uptake hydrogenase both in heterocysts and in unicellular forms is an integral thylakoid membrane protein. The enzyme feeds in electrons from H_2 either to a cytochrome (b or c) or directly to plastoquinone. The electrons are then transferred to the cytochrome b,f complex commonly utilized for photosynthesis and respiration on the thylakoids and are then allocated to either the respiratory complex IV or photosystem I (Eisbrenner et al., 1981). The enzyme is activated by the thioredoxin system on the thylakoids (Dai et al., 1992).

Attempts to find the genes coding for uptake hydrogenase by heterologous DNA-probing or by PCR consistently failed in the past. The genes have recently been found per chance in a screening program for DNA that undergoes rearrangement during heterocyst differentiation and prior to expression (Matveyev et al., 1994; Carrasco et al., 1995) similar to *nif* genes in *Anabaena* 7120. Late during heterocyst differentiation, a 10.5 kb

DNA segment is excised between 16 bp direct repeats that flank the element within the *hup*L gene coding for the larger subunit of this [NiFeS]-hydrogenase. The site specific recombinase XisC is encoded on this 10.5 kb element. The genes *hup*L and partly *hup*S have been sequenced (Carrasco et al., 1995).

Cyanobacteria possess at least a further hydrogenase, the so-called reversible or bidirectional enzyme, catalyzing both H_2-uptake (with PMS or MB) and the $Na_2S_2O_4$ and methylviologen (=MV)-dependent evolution of the gas (Houchins, 1984, Papen et al., 1986). This enzyme occurs both in heterocysts and vegetative cells of heterocystous cyanobacteria and also in unicellular forms. The enzyme is not activated by thioredoxin but its activity levels are enhanced by anaerobic incubation of the cells. Immunogold-labeling experiments with antibodies raised against the enzyme from *A. nidulans* indicated its location at or close to the cytoplasmic membrane (Kentemich et al., 1989). As the H^+-gradient is directed outwards in cyanobacteria and as the enzyme has a high affinity for H_2, it probably serves in recycling the gas at the periplasmic face of the cytoplasmic membrane.

The gene set coding for the bidirectional hydrogenase has now been localized on a 8.9 kb segment from *A. variabilis* (Schmitz et al., 1995). The cluster contains the two structural genes *hox*H and *hox*Y of this NiFeS-hydrogenase which, surprisingly, show strong homologies to the corresponding genes of the $NAD(P)^+$-reducing hydrogenases from *Alcaligenes eutrophus, Desulfovibrio fructosovorans* and the MV-reducing enzymes from archaebacteria. The 8.9 kb segment also contains the two genes *hoxU* and *hox*F genes coding for the diaphorase part of the $NAD(P)^+$-reducing hydrogenases. This gene cluster is interspersed with two open reading frames and with an 0.95 kb apparently non-coding region in an unusual way. Their function, if any, and the transcription units in this gene cluster have not yet been elucidated. The findings that *A. variabilis* (and also *A. PCC 7120, A. cylindrica* and *Anacystis nidulans*) possess the genes coding for such a hydrogenase is somewhat surprising, since extracts from cyanobacteria have never been described to catalyze a NAD(P)H-dependent H_2-evolution or a H_2-dependent reduction of $NAD(P)^+$ and since these organisms do not grow photoautotrophically with H_2 as reductant. Both NAD(P)H-dependent H_2-evolution (Schmitz et al., 1995) and H_2-uptake with $NAD(P)^+$ (Schmitz, unpublished) could now be demonstrated in extracts from *Anacystis nidulans*. Protein sequences of digests obtained from the purified bidirectional hydrogenase from *A. nidulans* matched with amino acid sequences translated from the DNA codons of the A. *variabilis* gene, leaving no doubt that the 8.9 kb segment encoded the bidirectional hydrogenase (Schmitz et al.. 1995). These findings rule out earlier suggestions that the electron acceptor for this hydrogenase is a low potential cytochrome (Morand et al., 1994).

Cyanobacteria might possess further hydrogenases. As said, the uptake enzymes from heterocysts and from the unicellular *A. nidulans* are presumably not related to each other. G. Smith and coworkers (Ewart and Smith, 1989, Ewart et al., 1990) characterized a soluble hydrogenase from *A. cylindrica*. The larger subunit (50 kDa protein) was reported to catalyze the dithionite and MV dependent H_2-evolution without the involvement of the smaller subunit which would be rather unusual for hydrogenases. The smaller subunit (42 kDa protein) was reported to catalyze the tritium exchange reaction, also alone. The gene coding for this smaller subunit was found to represent a new class of hydrogenases (Wu and Mandrand, 1993). Recent evidence suggest that the 42 kDa protein has sequence homologies to amino acid transferases (de Zoysa and Danpure, 1993; Ouzonis and Sander, 1993). The tritium exchange reaction may represent an interesting artificial side reaction of this enzyme otherwise unrelated to hydrogenases.

C. Cyanobacterial H_2-formations and potential applications in solar energy conversions

There is continuous interest to exploit cyanobacteria for solar energy conversion programs. Cyanobacteria have the simplest nutrient requirements in Nature. They thrive on photosynthetically in simple inorganic salt media and can meet their N-demand by N_2-fixation. It is the hope that the photosynthetically generated "reducing power" can be coupled to hydrogenases or nitrogenases to efficiently produce molecular H_2. Up-to date, formations by cyanobacteria are usually low, perhaps 1 µmol/(h x mg dry weight of cells) (Morand et al., 1994). Rates can partly be enhanced by immobilizing the cells (Gisby et al., 1987). However, the system suffers from its inherent instability, since H_2-production proceeds maximally few days. In addition, the solar enery conversion efficiency is generally under 1 %. Striking exceptions to this have been reported for the marine strain Miami BG7 (Mitsui and Kumazawa, 1977) and for the non-heterocystous *Lyngbya* (Kuwada et al., 1988) which showed high and sustained H_2-productions with a solar energy conversion efficiency of some 8 %. It is not yet clear whether any biological material can ever compete with the photovoltaic or any other established system in the generation of fuels.

In the isolates just mentioned and also in all other generally studied cyanobacteria (*A. variabilis, A.. cylindrica, A.* 7120), all H_2-formations come from nitrogenases. The maximal rate reported for H_2-formation by hydrogenase in *Anabaena* was 2 µmol x h^{-1} and g dry weight^{-1} (Hallenbeck et al., 1981) and thus extremely low. The situation may, however, be different in *Oscillatoria limnetica* where H_2S can serve as the photosynthetic electron donor (Belkin and Padan, 1978). H_2-production by fermentation has also been reported to occur in some cyanobacteria (Heyer et al., 1989; Heyer and Krumbein, 1991; van der Oost et al., 1989). The physiological function may be to dispose of excess of reductant under strict anaerobic conditions. It is not yet clear whether the pathway of this formation involves pyruvate:formate lyase and formate:hydrogen lyase as in the Enterobacteriaceae. Alternatively, H_2 could be generated from NAD(P)H and the bidirectional hydrogenase. The rates of this NAD(P)H-dependent formation in crude extracts from *A. nidulans* were, however, only about 1 % of the $Na_2S_2O_4$ and MV dependent activity (Schmitz et al., 1995) and thus very low. Clearly, H_2-production by fermentation in cyanobacteria cannot be exploited for solar energy conversion. It had been pointed out earlier that it is energetically more favourable to ferment carbohydrates to CH_4 than to H_2 (Bothe, 1982).

To maximize H_2-photoproductions catalyzed by nitrogenase in the autotrophic cyanobacteria, genetic manipulations of the H_2-utilizing hydrogenases seem to be prerequisital. Both the bidirectional hydrogenase of the cytoplasmic membrane and the uptake hydrogenase of the thylakoids appear to function in the utilization of the gas. Recent work with hydrogenase mutants from *A. variabilis* (Mikheeva et al., 1995) showed that H_2-productions can, indeed, be enhanced by affecting these enzymes.

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THE NIFO GENE PRODUCT IS RESPONSIBLE FOR THE ABILITY OF *Azotobacter vinelandii* TO SIMULTANEOUSLY ASSIMILATE NITRATE AND N_2

BOSCH, R.,[1] F. RODRÍGUEZ-QUIÑONES,[1] P. CARNICERO,[2] AND J. IMPERIAL [1,2]
(1) I.E.A., CSIC-UIB, Ctra Valldemossa, km 7.5, 07071 Palma, Spain;
(2) Lab. Microbiología, ETSI Agrónomos, Univ. Politécnica, 28040 Madrid, Spain

The *nifO* gene is part of the *nifBfdxNnifOQ* operon of *Azotobacter vinelandii*, and appears to be unique to this organism, since it shows no homology to *nif* genes from other diazotrophic microorganisms[1,2]. Mutations in *nifO* do not have any effect on nitrogenase activity or diazotrophic growth of *A. vinelandii* under normal, Mo-sufficient conditions [2]. However, NifO- mutants showed a clear Nif- phenotype when growth occurred in the presence of nitrate. Contrary to most diazotrophs, *A. vinelandii* strain OP does not significantly repress nitrogenase when assimilating nitrate, and nitrogen fixation contributes to growth yield in the presence of nitrate. However, in NifO- mutants grown on nitrate, nitrogenase activity disappeared. At the same time, nitrate reductase activity increased three-fold with respect to the wild type. *In vitro* assays and immunoblot analysis of nitrogenase components showed that dinitrogenase reductase is inactivated, and that the levels of nitrogenase structural polypeptides decrease under these conditions. This decrease could not be explained by a diminished transcription of nitrogenase genes alone. The increase in nitrate reductase activity in NifO- mutants might be directly responsible for the Nif- phenotype, both through increased nitrite levels, which inactive dinitrogenase reductase, and through increased ammonia levels, which repress *nif* transcription. We propose that the lower nitrate reductase levels of the wild type (NifO+) under nitrate are due to a NifO-catalyzed transformation of Mo into an iron-molybdenum cofactor precursor, which makes Mo unavailable for other reactions, such as synthesis of nitrate reductase Mo-cofactor. This idea is supported by: (1) the effect of NifO on heterologous Mo-dependent enzymatic systems. NifO inhibited molybdate reductase activity in *Escherichia coli* and respiratory nitrate reductase in *Klebsiella pneumoniae*; and (2) the specific *in vitro* interaction observed between NifO and NifQ. Taken together, these observations support a model in which NifO and NifQ act coordinately in an early nitrogenase-specific transformation of molybdenum. Supported by DGICYT (PB91-0120).

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NITROGEN REGULATION OF VNFA AND ANFA FUNCTION IN *Azotobacter vinelandii* ANALYSED BY CHIMERA FORMATION

DRUMMOND, M., FRISE, E., GREEN, A. AND JACOB, J.
BBSRC Nitrogen Fixation Laboratory, University of Sussex, Brighton BN1 9RQ, UK

Transcription of *nif* genes in the proteobacteria requires RNA polymerase containing sigma-54, which in turn needs an additional activator protein for open complex formation. These activators comprise a C-terminal DNA-binding domain, a catalytic central domain, and a regulatory N-terminal domin. Transcription of the three nitrogenase systems of *Azotobacter vinelandii*, encoded by the *nif*, *vnf* and *anf* genes, is driven by the activators NifA, VnfA and AnfA respectively. These differ in their promoter specificity and in that AnfA requires dinitrogenase reductase for its activity.

We have constructed genetic chimeras using *in vivo* recombination between the *vnfA* and *anfA* genes of *A. vinelandii*, and examined their properties in *K. pneumoiae*. Dependence on dinitrogenase reductase was found to be a function of the N-terminal domain of AnfA, and promoter specificity, as expected, a function of the C-terminal domain. In the course of this work, we also found that the activity of both VnfA and AnfA was repressed by ammonium. Removal of the N-terminal domains relieved ammonium repression in both proteins, implicating the N-terminal domains in the response to ammonium.

Two VnfA/AnfA chimeras with junctions in the central domain were susceptible to ammonium repression, but the most active was not. This differred from the ammonium-repressible chimera which most resembled it at only four psitions distributed over nine residues, three of the differences involving charge changes in what structure prediction identifies as a surface loop. We speculate that the inhibitory effect of the regulatory domain, which is normally relieved by ammonium deprivation, is transmitted to the catalytic domain by a mechanism involving this surface. The regulatory domains of VnfA and AnfA were exchanged by engineering restriction sites at analogous positions in the Q-linkers joining them to the central domains. This yielded partially ammonium-repressible chimeras, showing that signal transduction between the disparate N-terminal and central domains was possible, although less effective than in the native proteins. The chimera carrying the regulatory domain of AnfA showed weak dependence on dinitrogenase reductase, whereas that carrying the catalytic and DNA-binding domains of AnfA showed none, confirming the involvement of the regulatory domain of AnfA in signal transduction from the enzyme.

CHARACTERIZATION OF PSEUDOREVERTANTS OF A *nifU* MUTANT OF *Azotobacter vinelandii*

By HILL, S., HE*, L. AND KENNEDY*, C.
BBSRC Nitrogen Fixation Laboratory, University of Sussex, Brighton, UK
BN1 9RQ, UK and *Department of Plant Pathology, College of Agriculture,
University of Arizona, Tucson, AZ 85721, USA

The *nifS* and *nifU* gene products are now thought to be involved in formation and/or insertion of Fe-S clusters in both nitrogenase components (Zheng et al. 1993). We observed that both *nifS* and *nifU* mutants of *A. vinelandii* grew and reduced acetylene better in an atmosphere of 2% O_2 than in air on N-free agar or liquid medium. The mutant carrying a characterized deletion mutation in the *nifU* gene gave rise to 'pseudorevertant' Nif^{+} strains at a frequency of about 5 x 10^{-8}. The colonies were slightly green in color which is similar to mutants carrying Tn5 mutations in a gene identified as *cydR* in previous work (Kelly et al. 1990). *cydR* was within a region located 1 to 2 kb upstream of the *cydAB* genes; *cydR* mutants were Nif^{+} in air, Nif under microaerobic conditions, and slightly green in color; in addition, these mutants overproduced cytochrome *d* terminal oxidase and showed increased respiratory rates compared to wild type when grown in air. Genetic linkage experiments with the *nifU* pseudorevertants indicated that the mutations are closely linked to the *cydAB* region. Also, introduction of the *cydR::Tn5* locus into the *nifU* mutant strain resulted in Nif^{+} transformants.

Cytochrome *d* production during derepression for nitrogenase activity after growth on fixed N was higher in the pseudorevertants than in wild type. Expression of a *cydB-lacZ* fusion introduced into a pseudorevertant strain indicated that O_2 regulation of *cydAB-lacZ* expression was not impaired in the *nifU* and pseudorevertant backgrounds, and overproduction of cytochrome *d* in the pseudorevertant was not paralleled by the overexpression of *cydAB-lacZ*. Thus, discounting the possibility that cytochrome *d* has an auto-regulatory function, some other post-transcriptional or post-translational event results in higher activity of cytochrome *d* terminal oxidase.

The ability of *cydR*-like mutants to enable the *nifU* mutant to grow on N_2 in air has two possible explanations: 1) Nitrogenase enzyme is in similar states of 'maturation' in both aerobic and microaerobic cells; this partially active enzyme is more sensitive to oxygen inhibition than wild type enzyme. 2) Under microaerobic conditions, another cell protein can fulfill the function of NifU; this protein is more abundant or active in microaerobically grown cells than in air-grown cells.

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ISOLATION AND CHARACTERIZATION OF A *CYD⁻* MUTANT OF *KLEBSIELLA PNEUMONIAE*

JUTY, N.S.[1], ANTHONY C.[1] AND HILL, S.[2]
[1]Biochemistry Dept., University of Southampton, Southampton, SO16 7PX UK
[2]Nitrogen Fixaton Laboratory, University of Sussex, Brighton, BN1 9RQ UK

The *d*-type oxidase (cytochrome *bd'*) of *K. pneumoniae* has a very high affinity for oxygen, and is present during microaerobic nitrogen fixation as the sole oxidase (Smith et al., 1990). An *E.coli* mutant lacking this oxidase and carrying the *nif* genes of *K. pneumoniae* failed to show microaerobic nitrogenase activity, suggesting that it has an essential role in this activity (Hill et al., 1990). We report the construction of a *cyd* mutation in *K. pneumoniae* to test this hypothesis.

Degenerate primers, based on the *cyd* sequence of *E.coli*, were used to amplify the *K. pneumoniae cydA* gene by the polymerase chain reaction. This gene was mutated by insertion of the omega fragment, and a suicide vector (pMM46; Paul, Merrick, 1989) carrying the mutation was used to inactivate the chromosomal *cydA* gene of *K. pneumoniae*. This mutation has been confirmed by Southern hybridizations and by spectral analysis of whole cells grown in conditions suitable for induction of the oxidase in wild-type bacteria. The *cyd* mutant lacked cytochrome *bd'*, and was unable to utilize fermentation products to support microaerobic nitrogenase activity. Analysis of substrates able to support this activity in *K. pneumoniae*, but not in the mutant, suggests that oxidation of the fermentation product formate by way of cytochrome *bd'* is essential for the production of the ATP required for microaerobic nitrogen fixation.

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APPLICATION OF DIODE ARRAY SPECTROPHOTOMETRY FOR THE ANALYSIS OF BACTERIAL CYTOCHROMES *IN VIVO*

KAVANAGH E.P (1), CALLIS J.B. (2), HILL S. (1).
(1) Nitrogen Fixation Laboratory, at the University of Sussex, Brighton, BN1 9RQ UK.
(2) Department of Chemistry, University of Washington, Seattle, WA 98195,USA.

Respiration in organisms capable of aerobic N_2 fixation has at least two functions, they are: 1) to provide adequate ATP for nitrogenase activity and 2) to remove excess O_2 as part of the mechanism to protect nitrogenase from inactivation by O_2. The ability of the obligate aerobe *Azotobacter vinelandii* to fix N_2 under a wide range of rates of O_2 supply involves respiratory adaptation, which is not fully understood (for review see Hill 1992). We have investigated the potential of diode array spectrophotometry for the non-invasive identification and quantification of cytochrome components of the respiratory chain during growth of aerobes, particularly with regard to *Azotobacter vinelandii.*

Measurements *in situ* were made using a Hewlett Packard diode array spectrophotometer incorporating a modified tungsten light source and electronic shutter. Light was transmitted to and from the sample via a dual mode fibre optic bundle. Turbid samples (biomass between 0.1 to 1.1 mg protein. ml^{-1}) were subject to spectrophotometric examination in a cuvette or in the incubation chamber of a Clarke O_2 electrode. Furthermore, an adequate signal was obtained from *in situ* spectrophotometry in a chemostat vessel (0.16 mg protein ml^{-1}).

The advantages of our use of diode array spectrophotometry compared to the use of dual beam spectrophotometry (Pronk et al 1993) for *in situ* measurements include the following : 1) measurements are unaffected by ambient light, 2) the transitions between fully oxidised and the fully reduced spectra (2nd derivatives) can be recorded separately. They clearly revealed an isosbestic point for *A.vinelandii* cytochrome *d* at 642 nm, and 3) the almost simultaneous multi-wavelength measurements allows an assessment of the cytochrome complement and the oxidation states upon changes in external parameters, eg the supply of O_2.

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GENETICS OF NITROGEN FIXATION IN A METHANOGENIC ARCHAEON

KESSLER, P.S., BLANK, C., AND LEIGH, J.A.
Dept. Microbiology, Univeristy of Washington, Seattle, WA 98195, USA

We have initiated a study of the *nif* structural and regulatory genes in the methanogenic archaeon *Methanococcus maripaludis*. We constructed the transposon, Mu*d*Pur, containing the right and left ends of Mu, a chloramphenicol resistance gene for selection in *E. coli*, and a puromycin resistance gene for selection in *Methanococcus*. We used Mu*d*Pur to mutagenize a 15.3 kb λ clone of *M. maripaludis* DNA containing *nifH*. Insertions were obtained that spanned the 15.3 kb insert with a high degree of randomness.

Twelve mutagenized λ clones were introduced into *M. maripaludis* by transformation. Genomic DNA from each of the transformants was cut with restriction enzymes, run on a gel, and probed with an oligonucleotide hybridizing within the *nifH* gene. Mobility shifts showed that the mutagenized DNA had replaced the wild type region, apparently by double homologous recombination. In addition, λ vector DNA could not be detected. Thus, a series of transposon insertions were obtained throughout the 15.3 kb *nifH* region.

Nitrogen fixation was assayed by measuring growth (OD) on N_2 as the sole nitrogen source. The absence of any nitrogen source (argon atmosphere) was used as a negative control, and medium supplemented with NH_4^+ was used as a positive control. Final ODs revealed a clear distinction between strains that were able to grow on N_2 and those that were not. OD measurements were confirmed by assaying total cell protein. In this way, an 8 kb region in which mutations resulted in a Nif$^-$ phenotype was identified. Sequence analysis has so far led to the identification of seven genes in this region, *nifH*, *glnBi*, *glnBii*, *nifD*, *nifK*, *nifE*, and *nifN*.

The *nifH* gene was analyzed phylogenetically. The *M. maripaludis nifH* gene clustered with the *nifH* genes of the iron-only nitrogenases of the eubacteria. Nevertheless, *nifH*-dependent nitrogen fixation in *M. maripaludis* showed a distinct requirement for molybdenum.

In the future we are interested in studying how *nif* gene expression is regulated in methanogens. We would like to determine the function of the *glnB* genes.

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NITROGENASE SWITCH-OFF AND CHANGES IN CELLULAR ATP LEVELS IN *AZOTOBACTER VINELANDII* UNDER OXYGEN-STRESS.

LINKERHÄGNER K. AND OELZE, J.
Institut für Biologie II, Universität Freiburg, Schänzlestr.1, D-79104
Freiburg, Germany.

Steady state ATP levels as well as total levels of N fixed by diazotrophic chemostat cultures were determined after adaptation of *Azotobacter vinelandii* OP to different dilution rates (D) in the range of 0.05 to 0.3 h^{-1} and to ambient oxygen concentrations of 11.3, 68, and 135 µM. The results showed a strict proportionality between totally fixed N and ATP levels. Oxygen stress resulted in an immediate decrease of the cellular ATP pool: This decrease depended on the stress height. The ATP level decreased at a rate, which was about ten times as fast as D. When the oxygen stress was released, cellular ATP increased to the level before stress. The observed changes in cellular ATP levels were typical of oxygen stress with diazotrophic cultures. Mutant strain MK5 impaired in aerotolerant diazotrophic growth was able to grow diazotrophically at oxygen concentrations of up to 18-23 µM. After adaptation to 2.3 µM O_2, mutant MK5, its parent strain UW136 and the wild type strain OP were subjected to an oxygen stress of 38 µM O_2. The ATP pool of all of the strains decreased as above. When the oxygen stress was released the ATP pool recovered and nitrogenase was switched on. Adaptation from 2.3 µM to 38 µM O_2 was tested with diazotrophic chemostat cultures of strains OP, UW136 as well as MK5. Within a period of 9 h, the respiratory activity of UW136 increased by a factor of 2.8. The cell protein content of cultures of UW136 and MK5 decreased during the first 7 hours. Following this period, strain UW 136 attained a new constant steady-state biomass level, inferring reactivation of nitrogenase. With strain UW136, the specific rate of in situ nitrogen-fixation was 16.6 nmol of dinitrogen fixed per min and mg protein before stress and after adaptation to 38 µM O_2. According to the results shown above, oxygen stress led to a decrease in the ATP pool, reaching a minimum after 0.25 h of stress. Subsequently, the ATP level of UW136 started to increase. It reached the original value before stress after 5 h of incubation at 38 µM O_2. In contrast to its parent, mutant MK5 was washed out of the chemostat culture at 38 µM O_2. In agreement with its inability to form cytochrome *d*, MK5 was unable to significantly increase the respiratory activity. Most importantly, however, at 38 µM O_2 MK5 was unable to readjust the ATP pool to the level before stress. Since strain MK5 assumed to lack the uncoupled branch of the respiratory system was unable to readjust the ATP level, it may be concluded that, in spite of its assumed uncoupling, this branch has an indirect function in energy regeneration in accelerating the electron flow through the coupled initial part of the respiratory system. Regeneration of ATP, however, should be required for 'autoprotecrtion' of nitrogenase.

GENETIC CHARACTERIZATION OF AMMONIUM SENSING AND SIGNAL TRANSDUCTION IN *Azotobacter vinelandii*

MELETZUS, D., DOETSCH, N., GREEN, A., HE, L., RUDNICK, P.,
YAN, D., KENNEDY, C.
Department of Plant Pathology, College of Agriculture, The
University of Arizona, Tucson, AZ 85721, USA

The NifL proteins of *Azotobacter vinelandii* and *Klebsiella pneumoniae* inactivate NifA, the transcriptional activator of expression of other *nif* genes, in the presence of ammonium (and excess oxygen). Our aim is to understand how, in *A. vinelandii*, the signal of ammonium availability is transmitted and results in conversion of the NifL protein to its 'active' conformation in high ammonium and to its inactive form in ammonium deficiency. Genetic analysis indicated that the product of a *glnD*-like gene (initially called *nfrX*) was required for conversion of NifL to its 'inactive' conformation (Contreras et al, 1991). In contrast, recent studies of *K. pneumoniae* indicate that GlnD does not influence NifL activity in this organism (Merrick, Edwards, 1995). Based on the model of ammonium sensing and signal transduction in enteric bacteria, in which GlnD uridylylates the *glnB* gene product, PII, in the absence of ammonium, we searched for and found a *glnB* gene in *A. vinelandii*. Its sequence indicates a PII gene product with significant homology to other PII proteins, including a conserved Tyr at residue 49, the site of uridylylation. Interestingly, a gene encoding NrgA, previously identified in *Bacillus subtilis*, Saccharomyces, and arabidopsis and possibly involved in ammonium transport, was located downstream of *glnB*. In detergent-permeabilized cells grown in the absence of ammonium, ^{32}P-UTP labels PII and another protein of 40 kDa in size but not NifL. Attempts to introduce *glnB* deletion mutations into the *A. vinelandii* genome were unsuccessful; *glnB* appears to be an essential gene. Similarly, deletion mutations in *glnD* were lethal, while insertions in the 3' end of the gene were not; the latter were Nif$^-$, similar to the original *nfrX::Tn5* inserts which were all located at the 3' end. The only other mutations known to be lethal in *A. vinelandii* are those in *glnA* encoding glutamine synthetase; GS in *A. vinelandii* is inactivated in the presence of ammonium by its adenylylation. One hypothesis to explain the apparent lethality of *glnB* and *glnD* deletion mutations is that PII-UMP is required for deadenylylation of GS. Mutations at the adenylylation site of GS and deletions in *glnE* (encoding the adenylyl-transferase) are being constructed to test this hypothesis.

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REGULATION OF *RAHNELLA AQUATILIS* 333 *nif* GENE EXPRESSION MEDIATED BY THE NIFL PROTEIN AND THE *nif*LA PROMOTER

RUBIE C.
Institut für Genetik, Universität Bayreuth, 95440 Bayreuth, Germany.

In the present study various defined *nifL* mutants of *Rahnella aquatilis* 333* were designed by means of gentechnological methods in order to determine the function of the *nif*L gene of *R. aquatilis* 333 in the regulation of nitrogen fixation. One type of mutant was obtained by inserting a kanamycin resistence cartridge into a number of different restriction sites of the *R. aquatilis* 333 *nif*L gene; another type of mutant was obtained by deleting 136 base pairs in a defined area of the *nif*L gene of *R. aquatilis* 333. All mutants were physiologically investigated by means of the acetylene reduction assay in order to analyse the influence of temperature, partial pressure of oxygen and ammonia concentration on the ability of the mutants to reduce acetylene. Two different variations of the acetylene reduction assay were used to obtain information about the influence of these parameters on the formation of nitrogenase as well as on the activity of the formed enzyme. In additon, further results were obtained using a test system consisting of two compatible plasmids in a ß-galactosidase assay. The experiments performed on the homologous system in *R. aquatilis* as well as on the heterologous system in *E. coli* suggest that the *nif*L gene product of *R. aquatilis* 333 plays an important role in switching off *nif* gene expression of nitrogenase with a dependence on temperature, partial pressure of oxygen and ammonia concentration. Conseqently, it was concluded that the *nif*L gene product functions as a negative regulator in the *nif* gene expression of *R. aquatilis* 333. Moreover evidence was found that not only the *nif*L gene product but also the *nif*LA promoter of *R. aquatilis* 333 participates in the ammonia-dependent regulation of *nif* gene expression and that therefore *R. aquatilis 333* must possess a *ntr*-like system as exists in *Klebsiella pneumoniae*. The study of this *ntr*-like system with respect to its critical ammonia concentration was made possible by the construction of the defined *nif*L-deletion mutant mentioned above, whose *nif*L gene and corresponding *nif*L gene product have been destroyed but whose *nif*LA promotor has not been affected at all by the mutation. Therefore, in comparative physiological studies using the wildtype strain as a reference, this mutant allows a determination to be made of the border concentration of ammonia that effects this *ntr*-like system in a way that *nif* gene expression of *R. aquatilis* is switched off entirely.

* This strain has been previously described as Enterobacter agglomerans 333

CHARACTERIZATION OF *NIFHDK* IN *ACETOBACTER DIAZOTROPHICUS*

SEVILLA, M. AND KENNEDY, C.
Department of Plant Pathology, College of Agriculture
The University of Arizona, Tucson, AZ 85721 USA

Acetobacter diazotrophicus is a gram-negative N_2-fixing bacterium isolated from inside the roots and stems of sugarcane (1). We have initiated a program to identify and characterize *nif* and related regulatory genes in order to understand what factors influence nitrogen fixation in this unique habitat and to determine whether nitrogen fixed by *A. diazotrophicus* is significant to plant nutrition. Here we report the isolation, characterization and sequencing of *nifHDK*. A phage genomic library of A. diazotrophicus PAL5 was constructed using λ EMBL3. A 4.3 kb *Hind*III fragment carrying the *nifHDK* genes was identified by heterologous hybridization with the *nifHDK* genes of *Azospirillum brasilense*. The cloned *A. diazotrophicus nifHDK* fragment corresponded in size to a 4.3 kb hybridizing region of *Hind*III digested genomic DNA. The fragment was cloned into a sequencing vector, pSVB30, and nested deletions were made for sequencing.

The deduced gene product of the sequenced *nifH* gene of *A. diazotrophicus* showed high homology with the NifH proteins of other diazotrophs. The features common to all sequenced NifH proteins are also found in *A. diazotrophicus*. Seventy three percent of the *A. diazotrophicus* NifH amino acids are identical to those of *Rhizobium*. Data base searches and multiple sequence analysis showed that based on the NifH sequence, *A. diazotrophicus* is closely related to *Rhizobium*, *Bradyrhizobium*, *Rhodobacter*, *Azospirillum* and *Rhodospirillum*.

A mutation in the isolated *nifHDK* fragment was constructed by insertion of a cassette carrying *uid* and *aph* genes. Conjugation experiments between *E. coli* carrying the mutated fragment cloned in a suicide vector and *A. diazotrophicus* resulted in three transconjugants which are apparently unable to grow in N-free media. Further tests are currently underway to establish the Nif^- phenotype of these transconjugants. The Nif^- mutants will be used in inoculation studies of sterile sugarcane plants to evaluate the role of *A. diazotrophicus* in sugarcane nutrition.

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DOMAIN STRUCTURE OF *AZOTOBACTER VINELANDII* NIFL AND ITS INTERACTION WITH LIGANDS.

SODERBACK, E., EYDMANN, T., AUSTIN, S., DIXON, R.
Nitrogen Fixation Lab, Univ.Sussex, Brighton BN1 9RQ, UK

The autophosphorylated histidine conserved among autokinases in the two component family of transcriptional regulators is present in *Azotobacter vinelandii* NIFL. However, no *in vitro* phosphorylation of NIFL was found using [γ^{32}P]ATP or [γ^{32}P]GTP as potential phosphodonors. Varying concentrations of divalent metal ions (Mg^{2+} and Mn^{2+}) did not generate autokinase activity, even though the concentrations of these ions are important for autophosphorylation in members of the autokinase family (e.g. FIXL). The data therefore favour NIFL blocking NIFA activity via steric hindrance rather than via a phosphotransfer reaction.

Limited proteolysis of NIFL was used to characterise the domain structure of NIFL and effects of ligand binding. Stable trypsin digestion products were identified by amino-terminal sequencing. Proteolysis revealed two major domains of NIFL. The N-terminal domain remained stable during proteolysis. The C-terminal domain of autokinases in the two-component family have been suggested to possess two functionally separated regions, T_L and T_R, whose interaction may regulated the "catalytic activity" of these proteins, i.e. the autophosphorylation reaction (Parkinson, Kofoid, 1992). Two potential nucleotide binding motifs (glycine-rich sequences) are conserved in the T_R region, and mutations in these sequences disable the autokinase activity (Parkinson, Kofoid, 1992). Homologous sequences are present in NIFL and, although autophosphorylation apparently does not occur, we have evidence that adenosine nucleotides interact with the C-terminal domain, protecting sites against trypsin proteolysis. In the absence of ATP or ADP the C-terminal domain was relatively rapidly degraded by trypsin. ATP interaction with NIFL induced protection of the extreme C-terminus against trypsin attack. The resulting peptide represents the T_R region including the nucleotide binding sequences. In contrast, ADP protects more sites from proteolysis, rendering a peptide which also includes T_L, i.e. the complete C-terminal domain of NIFL. AMP, GTP and GDP do not protect the C-terminal domain. It therefore seems likely that ADP produces a conformational change in the C-terminal domain of NIFL required for its inhibitory interaction with NIFA.

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TWO GENES FOR THE PII PROTEIN CONTROL THE GLUTAMINE SYNTHETASE CASCADE IN *Escherichia coli*

VAN HEESWIJK, W.C.[1,2,3], MOLENAAR, D.[2,3], HOVING, S.[2,3], STEGEMAN, B.[2,3], KAHN, D.[4] AND WESTERHOFF, H.V.[1,2,3]
[1] E.C. Slater Institute, University of Amsterdam, Plantage Muidergracht 12, NL-1018 TV Amsterdam, The Netherlands; [2] Division of Molecular Biology (H5), The Netherlands Cancer Institute, Plesmanlaan 121, NL-1066 CX Amsterdam, The Netherlands; [3] Present address: Department of Microbial Physiology, Faculty of Biology, Free University, De Boelelaan 1087, NL-1081 HV Amsterdam, The Netherlands; [4] Laboratoire de Biologie Moléculaire des Relations-Plantes Microorganismes, CNRS-INRA, BP27, 31326 Castanet-Tolosan Cedex, France.

In enteric bacteria, assimilation of ammonia occurs primarily via the glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle. The activity of GS is regulated by a bicyclic cascade. GS is modified by adenylyl-transferase (AT) to produce the less active form GS-AMP. The latter can be deadenylylated by the same enzyme AT. The transferase activity is stimulated by the regulatory protein P_{II}, whereas the deadenylylase activity is enhanced by the uridylylated form of P_{II} (P_{II}-UMP). The modification of P_{II} is catalyzed by uridylyl-transferase (UT), which also has a deuridylyation activity. UT is regulated allosterically by 2-oxoglutarate and glutamine. Thus UT is a sensor for the nitrogen status of the cell. We are analyzing the glutamine sythetase regulatory cascade in *E. coli* in the light of the modular metabolic control theory (Kahn, Westerhoff, 1991).

In order to study the control of P_{II} on the GS cascade *in vivo,* a strain (WCH15) has been constructed with an inducible promoter upstream of *glnB*, the gene encoding P_{II}, at its wild-type chromosomal location. We have measured the kinetics of the adenylylation and deadenylylation of GS after a shift of ammonium concentration, at different cellular concentrations of P_{II}. In strain WCH15 grown on glucose-glutamine minimal medium (Ggln), the transient time of GS adenylylation after ammonium addition was independent of the cellular P_{II} concentration. Also when removing ammonium no effect on the transient-time of the GS deadenylyation was observed. Surprisingly, the *glnB* gene product had no control on GS adenylylation in this medium. However, in ammonium containing medium (GNgln) P_{II} exhibited a significant positive control on the deadenylylation-rate of GS-AMP. These intriguing results can be rationalised in the light of our finding of a second gene homologous to *glnB* in the *E. coli* chromosome. This gene, *glnB2* is repressed by ammonium. Thus in the absence of ammonium it can complement the *glnB* mutation.

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DINITROGEN EVOLUTION (DENITRIFICATION) BY NITROUS OXIDE REDUCTASE STUDIED BY SITE-DIRECTED MUTAGENESIS: COPPER LIGANDS, PROTEIN STABILITY, AND EXPORT COMPETENCE

ZUMFT, W.G. AND DREUSCH, A.
Dept. Microbiology, University Fridericana, D-76128 Karlsruhe,
Germany

N_2O reductase (NosZ) catalyzes the reaction $N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O$. The enzyme (EC 1.7.99.6) is part of bacterial denitrification which reverses N_2 fixation in the global N cycle. NosZ of *Pseudomonas stutzeri* is a Cu-containing enzyme. The ≈8 Cu atoms of the dimeric enzyme complex are organized presumably in two binuclear centers, Cu_A and Cu_Z (Zumft, Kroneck 1995). NosZ is located in the periplasm. A system for homologous *nosZ* expression was established. An internal 1555-bp *Sal*I-*Pvu*I fragment of *nosZ* was replaced by a kanamycin cassette. The resultant null mutant MK4211 was complemented by pSZ derived from pSUP104 by inserting *nosZ* on a 3500-bp *Xho*I-*Sma*I fragment. PCR-mediated mutagenesis was used for site-directed amino acid replacements. A R20D mutation within a conserved motif of the signal peptide caused transport incompetence of NosZ. An unprocessed enzyme without Cu accumulated in the cytoplasm. This places Cu insertion into NosZ at the cytoplasmic membrane or in the periplasm. The mutation H494C possibly targeted the Cu_Z site under loss of enzyme activity. An attempt to generate a Cu_Z protein by deleting the Cu_A center (C618 to P637) resulted in the loss of NosZ from the mutant strain. Since the transcript level of *nosZ* was unchanged versus the wild type, we assume a rapid degradation of the truncated protein. NosZ has 4 disulfide bridges (J. Riester, unpublished). The conserved C165 is not required for activity (Cu_Z) but contributes to a disulfide bridge essential for NosZ stability. The product of ORFL, downstream of *nosDFY* encoding the components for Cu incorporation, has the signature of a periplasmic disulfide isomerase. The Cu_A-binding site is a structurally conserved motif among 4 NosZ sequences and the subunit II of cytochrome *c* oxidase. Cu_A is located at the C-terminus of NosZ. Potential substitute ligands or evolutionary conserved exchanges were introduced. The exchanges C618D/V, C622D/V, H626G, and M629D/C caused the loss of NosZ activity; H583G retained ≈10% activity. These positions, thus, have an essential role in catalysis. The Cu_A site is important also for the periplasmic export of NosZ. The exchange C618V generated a NosZ species incompetent for transport; 90% of the enzyme remained unprocessed and accumulated in the cytoplasm. NosZ from H583G and M629C also was partially unprocessed. D580 may be part of the docking site for an electron donor analogous to findings with the Cu_A site of *Paracoccus denitrificans* (Lappalainen et al., 1995).

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WHERE IS NITROGENASE LOCALIZED WITHIN VEGETATIVE CELLS OF CYANOBACTERIA ?

BEKASOVA O.D.
Laboratory of Energy-Transforming Biocatalitic Systems, Bach Institute of Biochemistry, Leninsky prospect 33, Moscow 117071, Russia

In filamentous cyanobacteria nitrogen fixation takes place in specialized cells, known as heterocysts. In recent years some evidence to the presence of nitrogenase in vegetative cells has been accumulated. However, the position of nitrogenase in vegetative cells has not been defined. By means of the acethylene method we found nitrogenase activities in the preparations of phycobilisomes (PBS) isolated from *Nostoc muscorum*. Nitrogenase activity level in PBS was more than 10 times greater as compared to the original cyanobacteria grown on a nitrate medium. The increase of nitrogenase activity in the process of PBS isolation proves that the enzymic activity is not connected with contamination. This conclusion is also supported by the data, obtained on the PBS preparations from *N. muscorum* grown in a nitrate-free medium: the protein fraction enriched with 100-fold nitrogenase activity was isolated from such preparation of PBS. Redox properties of this fraction are similar to those of nitrogenase *in vitro*. Under illumination nitrogenase activity of isolated PBS is slightly inhibited and, on the contrary, it is highly stimulated in the original cyanobacteria. Our results suggest that PBS-bound nitrogenase arrangement in vegetative cells (PBS are not found in heterocysts). Besides, the obtained results indicate that properties of nitrogenase are differente in heterocysts and vegetative cells. The enzyme is associated with 34S-fragment, which is the ground of PBS. Nitrogenase may be located in "pockets", which are built up from the central cavities of stacked phycobiliproteins in rods or core of PBS. The presence of 10 % polyethylenglycol (m.w. 3000) in the medium for PBS isolation promotes the enzyme being kept in "pockets". This provides a reliable protection of the enzyme against oxygen. Thus, PBS in addition to well-known function of the light-harvesting antenna, are matrices for nitrogenase in vegetative cells.

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MOLECULAR BIOLOGICAL CHARACTERIZATION OF THE HYDROGENASES IN *ANACYSTIS NIDULANS*

BOISON G, SCHMITZ O, HILSCHER R, HUNDESHAGEN B, BOTHE H
Botanisches Institut der Universität zu Köln, Gyrhofstr. 15,
D-50931 Köln, Germany

The unicellular non-nitrogen fixing cyanobacterium *Anacystis nidulans* possesses at least two different hydrogenases, the bidirectional enzyme and the uptake hydrogenase, as described earlier (Kentemich et al. 1989). The bidirectional hydrogenase was purified in order to obtain protein sequence data. The sequences of proteolytic fragments of two subunits showed clear similarities to the deduced amino acid sequences of HoxH and HoxF of the bidirectional hydrogenase of *A. variabilis*, representing the large subunits of the hydrogenase and of the NAD(P)$^+$ reducing moiety, respectively (Schmitz et al. 1994).
A cosmid- and a l-gene bank of genomic DNA from *A. nidulans* were constructed in order to obtain information about the molecular biological organization of the hydrogenase genes. With the help of a PCR amplificate of an internal segment of *hoxH* from *A. nidulans* it should be possible to isolate corresponding clones representing the complete bidirectional hydrogenase gene cluster. Its characterization will be of interest especially in comparison to the organization of the bidirectional gene cluster of *A. variabilis*, where the four structural genes are seperated from each other by further ORF's of unknown function and a probably non-coding region, respectively (Schmitz et al. 1994). In collaboration with S. Shestakov (Moscow State University), mutagenesis of the bidirectional hydrogenase genes of both *A. nidulans* and *A. variabilis*, followed by complementation and transformation studies, will lead to a better understanding of the function of these enzymes. The molecular biological characterization of the uptake hydrogenase from *A. nidulans* is in progress. In contrast to the nitrogen fixing *Anabaena* 7120 where the uptake hydrogenase genes undergo a rearrangement during heterocyst differentiation (Carrasco et al. 1995), a rearrangement event in *A. nidulans* is unlikely. In addition, first hybridization experiments with the uptake hydrogenase genes from *A.* 7120 failed, indicating a lower degree of homology than in the case of the bidirectional enzyme.

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INTERACTIONS BETWEEN N_2 FIXATION AND ALTERNATIVE NITROGEN SOURCES IN *GLOEOTHECE*

CHENG J., HIPKIN C.R. & GALLON J.R.
School of Biological Sciences, University of Wales Swansea, Singleton Park, Swansea, SA2 8PP, UK

Gloeothece sp ATCC 27152, a unicellular cyanobacterium, can grow on N_2, NO_3^-, NO_2^- or NH_4^+ as sole nitrogen-source. However, N_2 fixation is confined to growth on N_2. Addition of 2mM NH_4Cl to N_2-fixing cultures of *Gloeothece* completely inhibited nitrogenase activity within 4h. This inhibition could be almost completely alleviated by simultaneously adding 3mM L-methionine-DL-sulphoximine (MSX), so appears to involve a product of NH_4^+ assimilation, probably glutamine (Thomas et al., 1982). In contrast, no inhibition of N_2 fixation by *Gloeothece* was seen within 7h of adding 2mM $NaNO_3$, though addition of 2mM $NaNO_2$ inhibited nitrogenase activity completely within this period. In marked contrast to its ameliorative effect on NH_4^+-mediated inhibition, MSX greatly enhanced the inhibitory effect of NO_2^- on N_2 fixation. In the presence of MSX, inhibition by NO_2^- was immediate. When cultures of *Gloeothece* were incubated under pure N_2, the inhibitory effect of NH_4^+ on nitrogenase activity was virtually identical to that seen in cultures incubated aerobically, but NO_2^- inhibited N_2 fixation by no more than 10% after 7h. Inhibition of nitrogenase by NO_2^- may therefore require O_2 and its mechanism of action differs markedly from that by which NH_4^+ inhibits N_2 fixation.

Exogenous NO_3^- or NO_2^- was taken up immediately by N_2-fixing cultures of *Gloeothece* though NH_4^+-grown cultures, transferred to medium containing NO_3^- or NO_2^-, consumed NO_3^- or NO_2^- only after a lag period of 2-4h. The assimilatory machinery for NO_3^- and NO_2^- in *Gloeothece* therefore appears to be NH_4^+-repressible rather than substrate-inducible, and is fully active in N_2-fixing cultures. In the presence of MSX, N_2-, NO_3^-- or NO_2^--grown cultures released NH_4^+ into the growth medium. Since little release of NH_4^+ was observed in cultures incubated with MSX in nitrogen-free medium under Ar, this demonstrates the ability of *Gloeothece* to reduce N_2, NO_3^- and NO_2^-, and also supports the idea that NH_4^+ is assimilated through the GS-GOGAT pathway.

NO_3^- and NO_2^- inhibited each other's uptake. NO_2^-, but not NO_3^-, also inhibited NH_4^+-uptake by N_2-fixing cultures of *Gloeothece*. On the other hand, NH_4^+ inhibited NO_3^--uptake completely, but NO_2^--uptake only partially. MSX blocked NH_4^+-uptake and also prevented NH_4^+ from inhibiting the uptake of NO_3^- or NO_2^-, so the true effector of this inhibition may be a product of NH_4^+-assimilation. Moreover, the mechanism by which NH_4^+ inhibits the uptake of NO_3^- and NO_2^- is different from that by which NO_3^- and NO_2^- inhibit each other's uptake, since MSX was without effect on the latter.

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FIT TO FIX-
SPECIALIZED CELLS FOR NITROGEN FIXATION IN A NON-HETEROCYSTOUS CYANOBACTERIUM

FREDRIKSSON C. & BERGMAN B.
Department of Botany, Stockholm University, 106 91 Stockholm, Sweden.

Trichodesmium is a filamentous non-heterocystous cyanobacterium, which has the extraordinary ability to fix nitrogen during the day without any apparent oxygen protection for nitrogenase. Even though colonies are present in oxic tropical/subtropical marine waters and evolve oxygen through photosynthesis, their maximum nitrogen fixation activity is reached at noon.

In order to find an explanation for this unusual nitrogen fixation behavior it has been of interest to localize nitrogenase within *Trichodesmium* colonies. Results of previous nitrogenase localization studies (Paerl, 1989; Bergman & Carpenter, 1991) were discrepant and therefore a large number of colonies were examined in this study. *Trichodesmium* colonies, composed of separate filaments were collected from their natural habitat in a diel cycle. The presence of nitrogenase was determined on cross-sectioned colonies using immunofluorescence/light microscopy and immunogold/transmission electron microscopy.

In all colonies examined nitrogenase was restricted to a subset of cells randomly distributed within the colonies. The frequency of nitrogenase-containing cells per section varied on a diel cycle. It was highest and constant ($P=0.05$) during the day with a 14% average of labelled cells. Within the labelled cells the concentration of nitrogenase varied, increasing in the morning reaching a maximum at noon and declining in the evening/night. These data indicate that there are a limited amount of cells or filaments specialized for nitrogen fixation within *Trichodesmium* colonies.

When cells with and without nitrogenase was compared ultrastructurally a number of structural differences were found. Cells containing nitrogenase had a denser thylakoid network, fewer cyanophycin granules and a smaller area of gasvacuoles per cell. Thus, like in heterocysts (Haselkorn, 1978) the cyanophycin granules are more or less absent and the thylakoids are extensive. However, unlike heterocysts they do not have a thick protective cell wall, the number of polyhedral bodies do not vary between labelled and unlabelled cells and cell division still occurs. The structural differences imply that there is a major metabolic change in these cells in order to accomplish a "nitrogen fixing" environment. Thus, Trichdesmium is a non-heterocystous cyanobacterium with differentiated nitrogen fixation cells.

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DEGRADATION OF NITROGENASE IN *GLOEOTHECE*

GALLON J.R.[1], READE J.P.H.[1], DOUGHERTY L.J.[1], PEDERSON D.M.[2] & ROGERS L. J.[3]
[1] School of Biological Sciences, University of Wales Swansea, Singleton Park, Swansea, SA2 8PP, U.K. [2] Department of Chemistry, CSU San Bernardino, 5500 University Parkway, San Bernardino, CA 92407-2397, U.S.A. [3] Institute of Biological Sciences, University College of Wales, Sir George Stapledon Building, Aberystwyth, SY23 3DD, U.K.

During aerobic growth of batch cultures of the unicellular cyanobacterium *Gloeothece* under alternating 12h light and 12h darkness, nitrogenase activity is confined to the dark period (see Gallon, 1992). Nitrogenase synthesis (measured as incorporation of radioactivity from $Na_2{}^{35}SO_4$) occurs only between 2h before and 6h after the onset of darkness, with the Fe- and MoFe-proteins synthesized in a ratio of about 2:1. Thereafter, both nitrogenase proteins disappear from the cells, suggesting that the enzyme is degraded. Degradation is especially rapid once cultures re-enter the light phase.

In vivo, no immunologically identifiable degradation products of nitrogenase are seen in cultures of *Gloeothece*. However, degradation of nitrogenase in crude extracts of *Gloeothece*, stored anaerobically, generated distinct breakdown products, at least some of which retain catalytic activity. Native Fe-protein (M_r 38 500) gave two products of M_r 34 700 and 32 900 respectively whilst native MoFe-protein (M_r 48 900 and 60 300) gave degradation products of M_r 43 700 and 56 300. Breakdown of nitrogenase was much less rapid in the presence of protease inhibitors, but occurred more rapidly when extracts were exposed to air, implying that at least part of the degradative process was stimulated by O_2. Exposure of extracts of *Gloeothece* to air for 15 min, whilst completely destroying nitrogenase activity, did not greatly stimulate degradation, suggesting that O_2-activated proteolysis may be more significant than specific targeting of O_2-inactivated protein in explaining nitrogenase degradation. Fe-protein from *K pneumoniae* was not degraded by aerobic extracts of *Gloeothece* that rapidly degraded the *Gloeothece* protein, suggesting that the nitrogenase-degrading system of *Gloeothece* is highly specific.

Protease inhibitors, added to cultures of *Gloeothece* 6h into the dark phase, were very effective in decreasing the disappearance of both nitrogenase proteins. These then persisted well into the light phase. Transfer of cultures to an atmosphere of O_2 also prevented degradation of nitrogenase. Exposure to O_2, or transfer to the light phase, stimulated conversion of the Fe-protein to its higher molecular weight form (Du, Gallon, 1993), which then appeared relatively resistant to proteolysis. No ubiquitinylated nitrogenase proteins were detected *in vivo*, even in the presence of protease inhibitors.

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INVOLVEMENT OF OXIDATIVE STRESS PROTEINS IN METABOLISM OF N_2-FIXING CYANOBACTERIA

JOHANSSON C.[1,2], JÄGER K.[2] & BERGMAN B.[1]
[1]Department of Botany, Stockholm University, S-10691 Stockholm, Sweden
[2]Botanisches Institut der TiHo-Hannover, Bünteweg 17D, D-30559 Hannover, Germany

Nitrogen-fixing cyanobacteria perform O_2-sensitive N_2 fixation and O_2-evolving photosynthesis simultaneously, and the defence against oxidative damage, including enzymes such as glutathione reductase (GR), which "recycles" antioxidants, likely contributes to protection of nitrogenase. Sensing of oxidative stress and regulation of several genes involved in the response, for instance *gor* which encodes GR, and which has recently been cloned from *Anabaena/Nostoc* PCC 7120 (Jiang et al. 1995), is achieved by the regulatory protein OxyR in *Escherichia coli* and *Salmonella typhimurium* (Christman et al. 1985, 1989, Storz et al. 1990).

Immunoblotting showed that the antibody directed against *Anabaena/Nostoc* PCC 7120 GR specifically recognised a 50 kDa protein also in *Anabaena variabilis* and *Nostoc* PCC 9229. After 10 min. induction, the band intensity tended to increase with increasing concentration of H_2O_2 (10 μM - 1 mM). In addition, heat shock at 42°C led to an increased detection of GR protein by immunoblotting in *A. variabilis* with time. Preliminary results using immunocytochemistry-transmission electron microscopy indicate that GR is present in both the N_2-fixing heterocytes/heterocysts and the photosynthesising vegetative cells.

Antibodies raised against the *E. coli* OxyR, recognised (a) specific protein(s) in *A. variabilis* (32.6 kDa) and *Anabaena/Nostoc* PCC 7120 (31.2, 34.1, 35.8 kDa) but not in *Nostoc* PCC 9229. Contrary to the situation in *E. coli* (Storz et al. 1990), the band intensity after treatment with increasing concentrations of H_2O_2 was comparable to or slightly more intense than that in the untreated control.

Nonetheless, using a digoxigenin-labelled *oxyR* gene probe under very low stringency conditions, no hybridisation was observed with any cyanobacterial DNA tested, including that from *A. variabilis* and *Anabaena/Nostoc* PCC 7120. This indicates that although an OxyR-like protein seems to be present in some cyanobacteria, the DNA homology is low.

Antibodies against the *E.coli* OxyR and the pAQ17 plasmid with the *oxyR* gene sequence (Christman et al. 1989, Storz et al. 1990) were kindly provided by Dr G. Storz. The technical assistance of C. Baß, H. Gommlich and J. Bunnenberg is acknowledged, as is the financial support of the Swedish Institute and the Deutsche Akademische Austauschdienst.

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MUTATIONAL ANALYSIS OF THE *fdxH* GENE, CLONING OF THE *dnaK* GENE AND IDENTIFICATION OF A NOVEL REPEATED DNA SEQUENCE IN *ANABAENA* SP.

MASEPOHL B., BACHEM S., GÖRLITZ K., KUTZKI C., POHL B., and BÖHME H.
Botanisches Institut der Universität Bonn, Kirschallee 1,
D-53115 Bonn, Germany

The *fdxH* gene from the filamentous cyanobacterium *Anabaena* sp. PCC 7120 encodes a [2Fe-2S] ferredoxin which is discussed to be the physiological electron donor to heterocyst nitrogenase. To verify this hypothesis, *Anabaena* 7120 *fdxH* null mutant strain BMB92 was constructed. For this purpose a 3.0 kb *Nhe*I fragment carrying the *fdxH* gene and a DNA region downstream of *fdxH* was replaced by a neomycin resistance cassette. The *fdxH* mutant was capable of slow diazotrophic growth and exhibited 20-25 % nitrogenase activity compared to the wild-type. These data confirmed that, the *fdxH* gene (and/or a gene located downstream of *fdxH*) plays an important role in nitrogen fixation but the *fdxH* gene is not essential for diazotrophic growth. It remains speculative whether PetF, another ferredoxin or flavodoxin can (partly) substitute for FdxH.

To analyze the role of chaperones in *nif* gene expression in filamentous cyanobacteria, we have cloned the *dnaK* (*hsp70*) gene from *Anabaena variabilis* ATCC 29413, which is the first *dnaK* gene identified in filamentous cyanobacteria. The motif EGGKP present in the DnaK proteins from chloroplasts and the unicellular cyanobacteria *Synechococcus* sp. PCC 7942 and *Synechocystis* sp. PCC 6803 but not in *E. coli* and other bacteria was also identified in *Anabaena* 29413 DnaK. Genetic analysis of the *dnaK* gene region is currently under investigation.

Reconstruction of the transposon Tn5-1087b induced mutation in *Anabaena* 7120 mutant strain YC16 (Het$^+$/Fix$^-$; Ernst et al., 1992) demonstrated that the Fix$^-$ phenotype of YC16 was not due to the Tn5-1087b insertion. Nucleotide sequence analysis of the DNA region carrying Tn5-1087b revealed the presence of 17 copies of a novel repeated DNA sequence designated LTRR (long tandemly repeated repetitive) sequence, 13 of which are completely identical. The LTRR sequence is 37 bp long and contains an inverted repeat sequence which might form a perfect stem-loop structure (7 bp stem, 3 bp loop). The LTRR sequences are separated by 31-42 bp.

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CHARACTERIZATION OF THE GENES CODING FOR THE BIDIRECTIONAL HYDROGENASE OF *ANABAENA VARIABILIS*

SCHMITZ O, BOISON G, HUNDESHAGEN B, BOTHE H
Botanisches Institut der Universität zu Köln, Gyrhofstr. 15,
D-50931 Köln, Germany

The filamentous cyanobacterium *Anabaena variabilis* can recycle the H_2 which is evolved during N_2-fixation by at least two different hydrogenases catalyzing the reaction $H_2 \rightarrow 2\ H^+ + 2\ e^-$. Enzyme activity measurements demonstrated that the so-called bidirectional hydrogenase is able to evolve H_2 *in vitro* in the presence of $Na_2S_2O_4$ and methylviologen as electron donor system in contrast to the uptake hydrogenase catalyzing solely the consumption of H_2. The specific amplification of an 1.35 kb segment of the bidirectional hydrogenase large subunit by PCR and screening of a genomic library of *A. variabilis* in phage λ allowed to isolate several overlapping clones revealing the organization of the bidirectional hydrogenase gene cluster (Schmitz et al., 1994). A 8.9 kb segment was characterized by subcloning and sequencing. In analogy to the NAD^+ reducing hydrogenase from *Alcaligenes eutrophus*, four structural genes, *hoxFUYH*, were identified by sequence similarities of the corresponding proteins ranging from 32 to 43% identical amino acid residues. A similar degree of sequence identity is pronounced when the small and the large subunit of the hydrogenase dimer (HoxYH) is compared to the methylviologen reducing hydrogenases of Archaea. HoxF and HoxU, constituting the diaphorase part of the enzyme, are more related to the corresponding subunits of the $NADP^+$ reducing hydrogenase from *Desulfovibrio fructosovorans* (HndC and N-terminal part of HndD) than to the *Alcaligenes* enzyme, showing sequence identities of 59 and 34%, respectively (Schmitz et al., 1995). The HoxF protein contains consensus sequences for the binding of NAD(P) and FMN and therefore possesses all essential prerequisites for a functional $NAD(P)^+$ reducing subunit. Enzyme activity measurements have been done verifying these molecular biological data. All the four structural hydrogenase genes are interspersed with two additional ORF's and a probably non-coding region which is unusual for a hydrogenase gene cluster. The function of these two ORF's, possibly in the regulation of hydrogenase activity, remains to be shown.

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FROM ENVIRONMENTAL TO DEVELOPMENTAL CONTROL OF NITROGEN FIXATION IN HETEROCYSTOUS CYANOBACTERIA: TWO DIFFERENT MO-NIF SYSTEMS EVOLVED FOR VEGETATIVE CELLS AND HETEROCYSTS OF *ANABAENA VARIABILIS* ATCC 29413.

SCHRAUTEMEIER, B., NEVELING, U., AND SCHMITZ, S.
Botanisches Institut der Universität Bonn, Kirschallee 1, D-53115 Bonn, Germany

Two different *fdxH* genes (*fdxH1*, *fdxH2*) have been isolated from the nitrogen-fixing, heterocystous cyanobacterium *Anabaena variabilis* ATCC 29413 as part of two different *nif* gene clusters, *nif1* and *nif2*. *fdxH1* encodes the [2Fe-2S] ferredoxin serving as the principal electron donor to nitrogenase in heterocysts. FdxH2 shares more amino acids and its oxygen sensitivity with the single FdxH from the nonheterocystous, filamentous cyanobacterium *Plectonema boryanum* PCC 73110 that induces nitrogenase early (≤ 3-4 h) after nitrogen depletion under anaerobic growth conditions in all vegetative cells. Likewise, *fdxH2* and the *nifHDK2* genes of *A*. 29413 were transcribed ≤ 4 h after nitrogen stepdown, exclusively under anaerobic growth conditions, and nearly 8 h before functional heterocysts appeared. *fdxH1* and *nifHDK1* transcription was not observed at this time. It occurred 8-10 h later under anaerobic or aerobic induction conditions for exclusive expression within heterocysts. No *fdxH2*/*nifHDK2*-transcription was detectable at any time under aerobic conditions. A Nif2 system was not detectable in *A*. 7120. Accordingly, under any growth conditions, nitrogenase activity was not observed before (pro)heterocysts appeared ≥ 10 h after nitrogen stepdown. These data reveal that in filamentous cyanobacteria two distinct *nif* gene clusters have evolved encoding different Mo-nitrogenases and related functions. Nif2 systems operate in vegetative cells of nonheterocystous and some, but not all, heterocystous cyanobacteria. They are environmentally regulated by the level of both combined nitrogen and oxygen. Nif1 systems probably have branched from an ancestral Nif2 system and evolved for an exclusive operation within heterocysts. As shown now, *nif1* expression has become an obligate late event in the developmental program of heterocyst differentiation, irrespective of aerobic or anaerobic growth conditions. Heterocyst-specific *nifHDK1* transcription may not be subject to (stringent) oxygen control, unlike *nifHDK2* transcription in vegetative cells. The regulatory mechanisms operating oxygen control of cyanobacterial *nif* genes are unknown. They may differ from the *fixLJ*/*nifLA*-based oxygen regulatory systems present in other nitrogen-fixing bacteria (Merrick, 1992). We are currently studying aspects of the differential regulation and oxygen sensitivity of the two Nif systems.

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ENZYMES INVOLVED IN HYDROGEN METABOLISM OF THE *CYANOBACTERIUM ANABAENA VARIABILIS* ATCC 29413: EFFECT OF HYDROGEN AND ORGANIC SUBSTRATE

YU, T.O.[1], SEREBRYAKOVA, L.T.[1], LINDBLAD, P.[2]
(1) Institute of Soil Science and Photosynthesis, Pushchino, 142292, Russia
(2) Department of Physiological Botany, Uppsala University, Villavagen 6, Uppsala, S-752 36, Sweden

Comparative study of development of uptake hydrogenase and nitrogenase activities in *Anabaena variabilis* has been performed depending on presence of fructose or molecular hydrogen. NH_4^+ -grown cells of *A. variabilis* exhibited low activity of hydrogen consumption [2-7 μmoles of $H_2 \cdot mg^{-1}$ (Chl *a*)$\cdot$ h^{-1}]. Hydrogen uptake was increased in more than 10 times when NH_4^+-grown cells were transferred to -N medium. Enhancement of hydrogen consumption activity was parallel with inducing of heterocyst differentiation and nitrogenase activity. Addition of fructose and further photoheterotrophic growth did not influence on development of uptake hydrogenase activity, but supported higher activities of both nitrogen fixation and hydrogen consumption (1.5-2 times) when culture entered at late exponential phase of growth. Presence of hydrogen in gas phase did not induce higher hydrogen oxidation in *A. variabilis* cells actively fixing nitrogen. Slight stimulatory effect of hydrogen (30-40%) on uptake hydrogenase activity was observed only in cells at early stages of nitrogenase induction.

FORMATION AND ACTIVITY OF NITROGENASE IN *RHODOBACTER CAPSULATUS* GROWING AEROBICALLY IN THE DARK.

KLEIN G., JAHN A., STEINDORF A., OELZE J.
Institut für Biologie II, Universität Freiburg
Schänzlestr.1,
D-79104 Freiburg, Germany.

Cultures of *Rhodobacter capsulatus* adapted to different oxygen concentrations in the dark, fix dinitrogen only at oxygen concentration of up to 1% of air saturation of the medium (1). Nevertheless, acetylene reduction, assayed under optimal conditions (anaerobiosis in the light), was detectable after growth of the organisms at oxygen concentrations of up to 20% of air saturation. Western-blots revealed that nitrogenase polypeptides were formed at oxygen concentrations of up to 30% of air saturation :However, ADP-ribosylation of Rc2 could not be detected. In order to confirm the observed inactivation under aerobiosis without modification of Rc2, mutant W 107 I of *R. capsulatus* reported to lack both the modifying (DraT) and the demodifying (DraG) enzymes (2) was included in the present studies. The mutant showed not only the same sensitivity of diazotrophic growth to oxygen as the wild type but also the same activities of acetylene reduction. In order to test whether ADP-ribosylation required stress conditions, i.e. a sudden increase in ambient oxygen, aerobic dark chemostat cultures adapted to 1% of air saturation were subjected to an oxygen stress of 50 % air saturation for 60 min. After 60 min of stress, acetylene reduction was decreased to 60% and the levels of the nitrogenase polypeptides were decreased in parallel to 75% of the values before stress. About 5 % of the latter can be attributed to the dilution of the chemostat culture taking at a rate of 0.05 h^{-1}. However, even under these conditions of oxygen stress, the presence of the modified form of Rc2 could not be detected. For control, cultures adapted to 1, 2, and 5% of air saturation were subjected to ammonium switch-off. Under these conditions, modification of Rc2 unequivocally occurred. From the present study it follows that inactive nitrogenase of aerobic of *R. capsulatus* is endowed with considerable stability against oxygen damage. However, in agreement with others (3), ADP ribosylation is not necessarily required for the inactivation of nitrogenase in *R. capsulatus*.

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STUDIES ON THE P_{II} PROTEIN IN *RHODOSPIRILLUM RUBUM*

JOHANSSON, M. AND NORDLUND, S.
Department of Biochemistry, Arrhenius Laboratories for Natural
Sciences, Stockholm University, S-106 91 Stockholm, Sweden

P_{II}, a trimer of identical subunits[1], is an important regulatory protein for the regulation of glutamine synthetase [2] and for regulation of NTRC dependent transcription from σ^{54} dependent promoters[3,4]. The most extensive studies on the P_{II} protein has been made in enteric bacteria were it is modified by uridylylation when the glutamine/α-ketoglutarate ratio in the bacteria is low[5]. We have previously reported on the gene sequence for *glnB*, encoding the P_{II} protein, from the photosynthetic diazotroph *Rhodospirillum rubrum*. The gene is localised in front of *glnA*, encoding glutamine synthetase, and shows high similarity with *glnB* from other organisms.

PCR amplification of a fragment containing *glnB* created a *NdeI* site at the 5´ end of *glnB*. The amplified fragment was cloned into T7lac expression vector pET16b (Novagen) fused in frame with plasmid DNA encoding $(\text{his})_{10}$ and the factorXa "restriction" protease site (Ile-Glu-Gly-Arg). *Echerichia coli* BL21(DE3)pLys (Novagen) harbouring pET16b/*glnB* was grown at 30°C to OD_{600}0.6 and induced with 1mM IPTG for 3 hours. The histidine fused P_{II} protein was purified with Ni^{2+} chelate affinity chromatography. A high yield of pure protein was obtained but attempts to remove the His tag with FactorXa has not yet been successful.

Samples from differently grown *R. rubrum* cultures were TCA precipitated and separated on 20% SDS-PAGE. Modified and unmodified forms of P_{II} were detected on Western blots, with two antibodies made against synthesised peptides, and developed by the ECL method. Thin cultures starved on nitrogen only exhibited modified form of P_{II}. When NH_4^+ was added P_{II} rapidly became demodified. Nitrogen fixing cultures at OD_{600} 0.7 showed a mixture of the two forms. Incubation in darkness of cultures grown either with ammonia or under nitrogen fixing conditions did not cause any detectable changes in the modification status of P_{II}.

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STUDIES ON THE ELECTRON TRANSPORT TO NITROGENASE I *RHODOSPIRILLUM RUBRUM*

LINDBLAD, A. AND NORDLUND, S.
Department of Biochemistry, Arrhenius Laboratories, Stockholm University, S-106 91 Stockholm, Sweden

The pathway generating reductant to nitrogenase has only been well established in *Klebsiella pneumoniae*. In this organism a pyruvate: flavodoxin oxidoreductase (*nifJ*) has been shown to transfer electrons from pyruvate to nitrogenase via a nitrogen fixation specific flavodoxin [1]. A pyruvate oxidoreductase from *Rhodospirillum. rubrum* has been purified and partially characterised [2]. The ability of a *nifJ* $^-$ mutant of *R. rubrum* to grow under nitrogen fixing conditions indicates that an alternative electron transport pathway is present in *R. rubrum*.

This study reports on the relation between the whole cell nitrogenase activity, the light intensity, the intracellular ATP/ADP ratio and the modification of the Fe protein (ADP-ribosylation). When light intensity was lowered to 32 and 10 %, no effect on nitrogenase activity and a slight increase in the ATP/ADP ratio were observed as compared to 100% light (960 $\mu E \cdot m^{-2} \cdot s^{-1}$). When the light intensity was lowered to 1%, there was a drastic decrease in nitrogenase activity but the ATP/ADP ratio remained high. In darkness no nitrogenase activity was observed and the ATP/ADP ratio decreased to 50 % of the ratio of illuminated cells. At 1% light the nitrogenase activity was 5% of that of the more illuminated cultures. This drastic decrease of activity is not only due to inactivation of the Fe protein by ADP-ribosylation, visualised by immunoblotting, as was observed in cultures incubated in darkness.

Initial experiments to investigate a NADH dependent nitrogenase activity were performed. The result shows that NADH supports nitrogenase activity when added to cells in which nitrogenase activity was inhibited by fluoroacetate.

These initial results support a model in which reduction of electron donors to nitrogenase in *R. rubrum* is coupled to the energization of the chromatophore membranes by photosynthesis and oxidation of NADH.

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POSTTRANSLATIONAL MODIFICATION OF DINITROGENASE REDUCTASE IN *Rhodospirillum rubrum* DURING NAD$^+$ SWITCH OFF.

NORÉN, A. and NORDLUND, S.
Department of Biochemistry, Arrhenius Laboratories,
Stockholm University, S-106 91 Stockholm, SWEDEN

Metabolic regulation of nitrogenase by modification of dinitrogenase reductase occurs in a number of phototrophs, cyanobacteria and some other diazotrophs. In the photosynthetic bacteria and some species of *Azospirillum* external effectors such as ammonia, glutamine,darkness, anaerobiosis and oxygen lead to a reversible switch off of nitrogenase activity (1) due to posttranslational modification of dinitrogenase reductase.
This phenomenon has been studied in detail in *R.rubrum* where the modification, ADP-ribosylation, is catalyzed by dinitrogenase reductase ADP-ribosylating transferase (DRAT) and the reverse reaction by dinitrogenase reductase activating glycohydrolase (DRAG)(1). In the reaction catalyzed by DRAT NAD$^+$ is the donor of the ADP-ribose moiety. NAD$^+$ added to nitrogen fixing cultures of *R.rubrum* causes a reversible decrease in nitrogenase activity (2). The effect of NAD$^+$ is more pronounced under lower light conditions and also there is a more pronounced effect in cell cultures having reached stationary phase(unpublished data). We have earlier shown that when nitrogen fixing *R.rubrum* is subjected to ammonia, glutamine or NAD$^+$ as switch off effectors, a drop in fluorescence of reduced pyridine nucleotides occurs (3). This drop would reflect a change in the redox state of the cell during switch off.
We have now, using Western techniques, shown that NAD$^+$ also causes a reversible modification of dinitrogenase reductase in *R.rubrum.* The modification is reversible with time and follows the change in nitrogenase activity. This leads us to believe that increases in NAD$^+$ concentration in the cell acts as a signal for activation of DRAT in *R.rubrum.* The control of DRAG activity is however still an open question.

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NITROGENASE ACTIVITY OF *RHODOBACTER CAPSULATUS* DURING SWITCHING OVER FROM LIGHT TO AMMONIUM LIMITATION OF CHEMOSTAT CULTURES

TSYGANKOV, A.[1], LAURINAVICHENE, T.[1], GOGOTOV, I.[1],
MIYAKE, J.[2] ASADA, Y.[2]
[1]Institute of Soil Science and Photosynthesis Russian Academy of Science, Pushchino, Moscow Region, 142292, Russia; [2]National Institute of Bioscience and Human Technology, 1-1-3 Higashi, Tsukuba, Ibaraki 305, Japan

A switching over from light limitation via double light-NH_4^+ limitation to NH_4^+ limitation occurred in chemostat cultures ($D=0.08\ h^{-1}$) of *Rh. capsulatus* strain B10 growing at different light intensities. Different growth conditions of the cultures in the photobioreactors, the culture layer being 13 and 200 mm, were investigated. In the latter case no NH_4^+ limitation (light saturation) occurred because average light intensity inside the photobioreactor did not exceed 30 W/m^2 even at maximal incident light intensity (200 W/m^2). Furthermore, these cells were subjected to very large though transient (due to stirring) light intensity variations, affecting their physiological state (nitrogenase activity, C/N and P content, photosynthetic pigments etc.). Nitrogenase activity measured as C_2H_2 reduction and H_2 photoevolution rates was maximal at ammonium limitation i.e. in photobioreactor with thin layer of culture and depended on light intensity only in the conditions of double limitation. In this photobioreactor nitrogenase activity of *Rh. capsulatus* was higher than in the photobioreactor with thickness of 200 mm even in double light and ammonium limiting conditions at equal average light intensity. *Rh. capsulatus* growing at light limitation had higher content of N, Bchl in RC and lower C/N ratio in both photobioreactors. The effects of photobioreactors construction on physiological state of culture are discussed.

CLONING AND CHARACTERIZATION OF *glt* GENE ENCODING GLUTAMATE SYNTHASE (GOGAT) INVOLVED IN THE EXPRESSION OF NITROGENASE ACTIVITY AND THE REGULATION OF N CATABOLISM IN *RHODOBACTER SPHEAROIDES* .

WU Y. Q., LU T., SONG H. Y.
Institute of Plant Physiology, Academia Sinica, 300 Fonglin Rd., Shanghai 200032, China

Fourteen GOGAT- mutants were independently isolated from *R. sphearoides* strain 601 by employing transposon Tn5-generated mutation and mutagenesis with NTG. Biochemical and Physiological analyses revealed that these mutants showed a defective pleiotrophic phenotype in nitrogen metabolism (Ntr⁻) and a Nif⁻ phenotype in RCVBN medium. With the frequency of $2x10^{-9}$ spontaneous revertants of the mutants were selected and shown that both GOGAT and nitrogenase activities as well as their ability to utilize several N source were restored. A cosmid named pLT27 carrying 26.5 kb of genomic DNA from *R. sphearoides* strain 601 were identified and shown to have a homological sequence with *Escherichia coli gltBD* genes and *Azorhizobium sesbaniae glt* gene. PLT27 was able to complement 12 mutants containing Tn5 insertions, which produced characteristic GOGAT-, Nif⁻ and Ntr⁻ phenotypes. 4.0-kb and 10.5-kb *Hin*dIII fragments from 26.5-kb insert DNA were subcloned in plasmid pRK415 to form chimera pLTRK271 and pLTRK272, respectively. Southern blot indicated that the specific positions of tansposon insertions of the mutants GT6, GT10 and GT11 mapped within a 7-kb *Eco*RI-*Hin*dIII fragment present on pLTRK272, which could complement mutants GT6, GT10 and GT11 but failed to complement other 9 mutants. Those of the 9 mutants mapped within the 4.0-kb *Hin*dIII fragments in pLTRK271, which only complemented the 9 mutants. A 11-kb *Eco*RI-*Hin*dIII fragments of *R. sphearoides* genome DNA contains genetic information corresponding to some structural or regulatory loci for GOGAT biosynthesis.

DEREPRESSIONAL EXPRESSION OF NITROGENASE BY PYRUVATE-INDUCED CULTURE OF *RHODOBACTER SPHEAROIDES* GLUTAMATE SYNTHASE (GOGAT⁻) MUTANT.

WU Y. Q., ZHU M. Z., QIU Z. AND SONG H. Y.
Institute of Plant Physiology, Academia Sinica, 300 Fonglin Rd., Shanghai 200032, China.

A GOGAT$^-$ mutant 204 of *Rhodobacter sphearoides* showed a Nif$^-$ phenotype in RCVBN medium containing Glu as a nitrogen source and malate as a carbon source (Song *et al.* 1984). It caused the appearance of nitrogenase activity measured by acetylene reduction and hydrogen production while shifting the cells to a pyruvate-containing medium without malate. The mutant also displayed high degree of nitrogenase activity even under 10-60 mmol/L of Glu in the culture supplemented with 20mmol/L of pyruvate (the molecular ratio of Glu to pyruvate could be more than 3:1)(Wu *et al.*,1992). SDS-PAGE showed that the mutant grown on pyruvate synthesized much more nitrogenase in comparison with grown on RCVBN. The Mo-Fe protein content in the mutant cells on pyruvate was higher than on malate by two times as measured by rock immunoelectrophoresis (Wu *et al.*, 1993). The analysis by High Performance Liquid Chromatography (HPLC) revealed that the intracellular Gln pool in the mutant was 16 times higher than in the parent strain because the break of GOGAT reaction. Much less ammonia was excreted into the medium when the mutant was cultured with pyruvate than with malate. Nitrogenase activity occurred concomitantly with the depletion of intracellular Gln in the mutant subjected to pyruvate induction. Moreover, the glutaminase activity was found in *R. sphearoides* cells. The specific activity of glutaminase was higher in mutant induced by pyruvate than by malate. The enzyme, which was inhibited by DON (6-diaxo-5-oxo-2-norleucine) but not MSX (L-methionine-DL-Sulfoximine), may be involved in the decomposition of intracellular Gln. The intracellular level of Gln could be the key effector to the feedback modulation of nitrogenase activity.

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ROLE OF THE *NTRB* AND *NTRC* GENES OF *RHODOBACTER SPHAEROIDES* IN THE REGULATION OF NITROGEN AND CARBON METABOLISM

ZINCHENKO V., BABYKIN M., GLASER V., SMIRNOVA V., ABDULLAEV Z., POPOV A., SHESTAKOV S.
Dept. of Genetics, Biology Division, Moscow State University, Moscow, 119899, Russia.

The *R.sphaeroides* mutant strain Drn12 which expresses nitrogenase activity constitutively with respect to ammonia has been investigated. This strain of complex phenotype grows normally on NH_4^+, glutamine or glutamate, but shows slow growth on N_2 and can not grow on histidine and NO_3^-. Glutamine synthetase (GS) activity and expression of *glnB-lacZ* are strongly reduced and are about 5% of that in wild type cells under derepression conditions. Complementation and sequence analyses revealed that Drn12 has mutation in *ntrC* gene resulting in the replacement of conservative Gly26 for Asp at N-terminus of NtrC. This mutation presumably leads to the alteration of the phosphorylation status of NtrC protein. The similar mutant phenotype with derepressed nitrogenase was obtained by insertion inactivation of *ntrB* gene (strain WZ45). This fact indicates that *ntrB* is not absolutely required for the regulation of nitrogen fixation and NtrC protein might be phosphorylated by "cross-talk" from other protein kinases. Nif-constitutive phenotype of Drn12 and WZ45 with respect to ammonia is associated with low level of expression of *glnBA* operon, and is due to the low rate of ammonia uptake and low level of GlnB protein which is a negative regulator of *nif*-gene expression. The mutant strain PO597 with insertion inactivation of *ntrC* is glutamine auxotroph in the cells of which the expression of *glnB-lacZ* and *nifH-lacZ* is strongly reduced under derepression conditions. These data indicate that expression of *glnA, glnB* and *nifH* depends on *ntrC* function. In addition, PO597 is unable to use pyruvate and the substrates metabolizing via pyruvate as a sole carbon source and is capable to grow only under aerobic condition. Thus, *ntrC* is involved in the control of carbon/energy metabolism. This permits to suggest that the regulation of nitrogen and carbon/energy metabolism may be interlinked on the genetic level in photosynthetic bacteria.

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EXPRESSION STUDIES OF THE INDOLE-3-PYRUVATE DECARBOXYLASE GENE FROM *AZOSPIRILLUM BRASILENSE* Sp245 AND ANALYSIS OF THE DOWNSTREAM DNA REGION

COSTACURTA, A., KEIJERS, V.
AND VANDERLEYDEN, J.F.A.
Janssens Lab for Genetics, Catholic Univ. of Leuven,
Willem de Croylaan 42, B-3001 Heverlee, Belgium

Azospirilla were isolated as nitrogen-fixing bacteria, associated with the roots of graminaceous plants. They were first studied in view of the possibility that bacterial nitrogen fixation is responsible for the beneficial effects observed on plants. Later studies accumulated evidence that increased plant growth after inoculation with *Azospirillum* is observed also in absence of nitrogen fixation (Bashan *et al.*, 1989). Some authors proposed the hypothesis that the phytohormone indole-3-acetic acid (IAA), produced in high amounts by *Azospirillum*, is the factor responsible for the morphological changes observed on inoculated plants and for plant growth promotion (Tien *et al.*, 1979) The *ipdC* gene of *Azospirillum brasilense*, coding for indole-3-pyruvate decarboxylase which is a key enzyme in IAA biosynthesis was cloned and sequenced (Costacurta *et al.*, 1994). The DNA region flanking the *ipdC* gene from *A. brasilense* was sequenced in an attempt to identify other genes involved in IAA production. Upstream from *ipdC* but with opposite orientation, genes homologous to glutamyl-tRNA synthetase (*gltX*) and cysteinyl-tRNA synthetase (*cysS*) of *E. coli* and *B. subtilis* were identified (Costacurta *et al.*, submitted). 100 bp downstream from the *ipdC* gene a partial *ORF* homologous to the putative sigma factor SCRP-27 from *E. coli* was detected. A mutant carrying a Tn5 insertion in this ORF was constructed by homogenotization. An interesting characteristic of the Tn5 insertion mutant obtained, was faster growth both in minimal and rich medium. Studies of a *ipdC-gusA* fusion revealed that the *ipdC* gene is not expressed in the constructed mutant. Analysis of the *ipdC-gusA* fusion in *A. brasilense* Sp245 wild-type revealed that the *ipdC* gene expression is enhanced in the presence of IAA and NaCl. Several auxin-regulated genes and cDNAs have been identified in plants and few genes from Agrobacteria. Only some of these IAA-induced genes have IAA specific induction while others appear to be responsive also to a number of other compounds such as cytokinin, salicylic acid, methyl jasmonate, heavy metals, wounding and environmental stress. The increase of *ipdC-gusA* expression by NaCl, which mimics increased osmotic pressure caused by pathogens infections, points towards such a kind of regulation.

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ISOLATION AND ANALYSIS OF THE *AZOSPIRILLUM BRASILENSE trpEG* OPERON, ENCODING ANTHRANILATE SYNTHETASE

DE TROCH, P., DOSSELAERE, F., KEIJERS, V., VANDERLEYDEN, J..
F.A.Janssens Laboratory of Genetics, K.U. Leuven, Willem de Croylaan42,
B-3001 Leuven, Belgium.

Indole-3-acetic acid production by *Azospirillum brasilense* can be enhanced by the addition of tryptophan to the growth medium or by overproduction of tryptophan (Reyders, Vlassak, 1979; Hartman et al. 1983; Cho et al. 1993). Tryptophan is synthesized from chorismate during which anthranilate synthetase (E.C. 4.1.3.27) converts chorismate into anthranilate. The genes involved are *trpE* and *trpG*, which encode component I and II of the anthranilate synthase. The *trpE* gene expression in *Escherichia coli* is tightly regulated by tryptophan and anthranilate synthetase, the TrpE enzyme is feedback inhibited by triptophan. By engineering the promoter or gene product, a strain with increased tryptophan synthesis should be obtained.

The *A.brasilense trpE* gene has been isolated by hybridization of a cosmid library of *A.brasilense* Sp7 with the *A.lipoferum trpE* gene (Ramschutz et al. 1992). The *A.brasilense trpE* gene is located on a 12 kb long *Eco*R1 restriction fragment, of cosmid pFAJ901. By restriction analysis, the *A.brasilense trpE* gene has been localized on a 3.1 kb long Pstl restriction fragment, which was subcloned into pUC18, generating pFAJ902. To confirm that pFAJ902 contains the *A.brasilense trpE* gene, the clone was transferred into an *E.coli trpE* deletion mutant, W3110 D*trpE*5, which was then able to grow on minimal medium without added tryptophan, indicating that tryptophan synthesis was restored. The DNA sequence of the 3.1 kb Pstl restriction fragment shows homology to anthranilate synthetase component I (*trpE*) and component II (*trpG*). The highest homology has been detected with the *R.meliloti trpE*(*G*) gene (Bae et al. 1989). However, another *trpG* gene has been isolated and sequenced in *A.brasilense* (Zimmer et al.1991). Both sequences are not completely homologous. The physiological meaning of the presence of two *trpG* copies in *A.brasilense* still has to be elucidated.

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IDENTIFICATION OF A N-REGULATED P_{II}-LIKE STRUCTURAL HOMOLOGUE IN *AZOSPIRILLUM BRASILENSE.*

deZAMAROCZY, M., PAQUELIN, A. AND ELMERICH, C.
Institut Pasteur, Département des Biotechnologies
Unité de Physiologie Cellulaire, CNRS URA 1300,
25, rue du Dr. Roux, 75724 Paris Cedex 15, France.

Glutamine synthetase (GS) is encoded by *glnA* and is a universal key enzyme of ammonia assimilation. In enteric bacteria, there are four proteins involved in the nitrogen regulation of *glnA* transcription. UTase acts as the primary nitrogen sensor. The small trimeric protein P_{II}, encoded by *glnB*, is a signal transmitter and co-ordinates the response of the Ntr system to the intracellular N-status. The two other proteins, NtrB a histidine kinase, and NtrC the transcriptional activator of *glnA*, belong to a two component regulatory system.

Regulation of ammonia assimilation in *Azospirillum brasilense* has some unusual features (de Zamaroczy et al. J. Bacteriol. (1993) 175, 2507 - 2515). The transcription of the *glnB-glnA* cluster depends on three different, selectively used and nitrogen-regulated promoters. NtrB / NtrC are not involved in transcriptional regulation of *glnB-A*. P_{II} participates in the repression of its own synthesis in response to N excess. In addition, *A. brasilense* is the only nitrogen fixer studied so far, in which *glnB* is an essential regulatory gene for nitrogen fixation.

In contrast with enteric bacteria, P_{II} is not involved in the regulation of reversible adenylylation of GS in response to ammonia availability. In addition, although *glnB::kan* null mutant strains are motile in liquid media and exhibit similar growth rate, colony shape and mixed pattern of flagellation, as the wild type strain, they are impaired in their swarming properties. In semi-solid media, the swarming distance is half that of the wild type strain Sp7, indicating that P_{II} contributes to the regulation of motility.

We investigated the N-regulated modification of P_{II} and its role in the regulation of nitrogen metabolism. Bacteria grown in N-free medium were labelled *in vivo* with tritiated uracil. A single band, corresponding to a 13 kDa polypeptide, was revealed by SDS-PAGE, in both the wild-type and several *glnB* null-mutant strains. No band was detectable in the presence of ammonia.

Western blot analysis, using two different anti-*A. brasilense* P_{II} antisera, revealed a polypeptide comigrating in SDS-PAGE with the tritiated polypeptide, both in the wild type and in *glnB* null-mutant strains. These results suggest that the 13 kDa polypeptide band corresponds to two comigrating proteins. The presence of two polypeptides in this doublet was confirmed by iso electro focusing electrophoresis. A single band was revealed, by anti-P_{II}-antisera, at pH 6.3, in the *glnB* null-mutant whereas, two bands were revealed at pH 5.7 and at pH 6.3, in the wild type strain.

Consequently, in agreement with the predicted size of the *glnB* gene product, a second P_{II}-like structural homologue, which is not a functional homologue of P_{II}, is produced by *A. brasilense*. We currently trying to identify the *glnB*-like gene by Southern hybridization and direct PCR amplification.

PHYSICAL AND GENETIC ANALYSIS OF AN 85-MDa PLASMID OF *AZOSPIRILLUM BRASILENSE* SP245

KATZY E.I., BORISOV I.V., PETROVA L.P.
Laboratory of Microbial Genetics, Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, Saratov 410015, Russia

Genetical studies on *Azospirillum* and their plasmids have been initiated in the early eighties. Since then, only a 90-MDa plasmid (p90) of *A. brasilense* Sp7 has been studied in detail (Croes et al., 1991, Onyeocha et al., 1990). It is still necessary to evaluate significance of other *Azospirillum* plasmids for the host bacteria and for the plant-bacterial interaction. We have concentrated on the analysis of an 85-MDa plasmid (p85) of *A. brasilense* Sp245. It was demonstrated that mobilizable derivatives of p85, p85-*Tn*5-Mob and p85-pSUP5011, can maintain in *Agrobacterium tumefaciens* and *Pseudomonas putida* plasmidless strains and in *A. brasilense* Sp7 but not in *Escherichia coli* strains. It was shown that p85 of Sp245 and p90 of Sp7 are stably coinherited whilst p115 of Sp7 disappeares from all the transconjugants of Sp7 (p85) tested. A number of *A. brasilense* Sp245 mutants with altered plasmid content were obtained. In the result of the analysis of these mutants and of the transconjugants of *A. tumefaciens* and *P. putida* harbouring p85-pSUP5011, we identified involvement of p85 into the indole-3-acetic acid (IAA) production. To obtain pure p85-pSUP5011 DNA of restriction quality a modified alkaline lysis procedure was used. *Eco*RI, *Bam*HI, *Bgl*II and *Hind*III restriction patterns of p85-pSUP5011 were studied. To identify p85 loci involved in IAA production, a plasmid gene bank of p85 was constructed. The bank is used for the enhancement of IAA production in *A. tumefaciens* GMI9023.

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CLONING AND SEQUENCEING OF A HOMOLOGOUS FRAGMENT OF *draT* AND *draG* GENES FROM *Azospirillum brasilense* Yu62

MA, L., LI, J.
Department of Microbiology, Beijing Agricultural University, Beijing 100094, P.R.China

The nitrogenase activity in *A. brasilense* is regulated by reversible ADP-ribosylation of dinitrogenase reductase as it is in *Rhodospirillum rubrum*. Two genes involved in this regulation system, *draT* and *draG* genes (encoding dinitrogenase reductase ADP-ribosyltransferase and activating glycohydrolase respectively), have been cloned and sequenced, but their promoter has not been found both in *A.brasilense* and *R. rubrum*. The *draTG* genes are contiguous and linked to *nifHDK* genes. (Fitzmaurice et al., 1989; Zhang et al., 1992)

Total DNA of *A. brasilense* Yu62, a strain isolated from maize in China, was digested with several restriction endonucleases. It then was probed with the *draTG* genes of *A. brasilense* Sp7 (4.0-kb EcoRI fragment). The *draTG*-hybridizing region was located on a 8.3-kb SalI fragment. This fragment was cloned by screening the gene library of *A. brasilense* Yu62 which was constructed in λEMBL3 vector. Further Southern hybridization demonstrated that *draTG* genes and *nifH* gene were located in a 5.0-kb fragment. These genes were sequenced respectively. A 3.0-kb EcoRI-KpnI fragment, containing *draTG* genes, has been completely sequenced on both strands. Analysis of the sequenced region revealed the 888-bp *draT* gene would encode a 33.7-kd protein and the 894-bp *draG* gene would encode a 32.4-kd protein. Compared with the same gene of. *A. brasilense* Sp7, *draT* shows 84% similarity and *draG* shows 96% similarity both at the amino acid level. An ORF immediately downstream of *draG* was also revealed.This 450-bp ORF would encode a 15kD protein just like the ORF in the same position in *R. rubrum*. Comparison of this ORF with that in *R. rubrum* shows 63% similarity at amino acid level. A 2.0-kb EcoRI fragment, which contains upstream region of *draT* and a portion of *nifH* gene, has been sequenced on one strand. The preliminary sequence is reminiscent of a putative promoter in the upstream region of *draT*, which is probably the promoter of *draTG*. This needs to be further verified.

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THE ORF1-*ntrB-ntrC* OPERON OF *Azospirillum brasilense* IS NEGATIVELY AUTOREGULATED.

MACHADO H.B.[1], YATES M.G.[2], FUNAYAMA S.[3], RIGO L.U.[3], STEFFENS M.B.R.[3], SOUZA E.M.[3], PEDROSA F.O.[3]
[1]Department of Pharmacology and [3]Department of Biochemistry, UFPR, C. Postal 19046, 81531-990 Curitiba, PR, Brazil.
[2]NFL-AFRC, Univ. of Sussex, Brighton BN1 9RQ, England.

Regulation of nitrogen fixation in *Azospirillum brasilense*, an associative diazotroph, is not fully understood. Regulation of *nif* expression in this organism appear to involve *nifA*, *ntrC* and *glnB* gene products (Pedrosa and Yates 1984, Liang *et al.* 1992, 1993). The sequence of the *A. brasilense ntrBC* genes has been reported but no promoter sequences were found upstream from the *ntrB* gene (Liang *et al.*, 1993).
We have sequenced a 6 kb DNA fragment from *A. brasilense* strain FP2 capable of complementing the Nif$^-$ and nitrate-dependent growth phenotypes of the *A. brasilense ntrC*$^-$ mutant FP9. This region showed two ORFs identified as the *ntrB* and *ntrC* genes. An additional ORF1 was found upstream from the *ntrB* gene, coding for a 36 kDa polypeptide, similar to the *nifR3* gene of *Rhodobacter capsulatus* and to the ORF1 of *Rhizobium leguminosarum* (Foster-Hartnett *et al.* 1993, Patriarca *et al.* 1993). Two other unidentified ORFs (ORF5 and partial ORF4) coding for hydrophobic polypeptides were also found flanking the ORF1-*ntrBC* genes. ΔORF1-*ntrBC*-, ORF1-, *ntrB*- and *ntrC-lacZ*-kanamycin *A. brasilense* insertional mutants named HDK1, HRK1, HBK1 and HCK1, respectively, showed decreased nitrogenase activities (ca. 50%) and failed to grow on nitrate as the sole N-source. Complementation of these mutants with plasmids containing the ORF1, *ntrB* and *ntrC* genes together or isolated, showed that the wild type phenotypes were only restored when both the *ntrB* and *ntrC* genes were active. Analysis of chromosomal *lacZ* fusions of mutants HDK1, HRK1, HBK1 and HCK1 suggests that the *ntrB* and *ntrC* genes are transcribed from a promoter located upstream of the ORF1. β-galactosidase activities of these mutants were markedly decreased by the presence of plasmids containing the *ntrC* gene suggesting that the ORF1-ntrBC operon is negatively regulated by NtrC. Supporting these results, two sequences resembling the cannonical σ^{70} promoter were found 24 and 64 bp upstream from the potential initiation codon of ORF1, respectively, and are overlapped by two sequences resembling NtrC-binding sites (Machado *et al.* 1995).

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THE *Azospirillum brasilense ntrA* GENE IS INVOLVED IN NITROGEN FIXATION, NITRATE ASSIMILATION, AMMONIUM UPTAKE AND FLAGELLAR BIOSYNTHESIS

MILCAMPS, A. (1), VAN DOMMELEN, A. (2), STIGTER, J.(1), VANDERLEYDEN, J. (2) AND DE BRUIJN, F.J. (1)
(1) Plant Research Laboratory, Michigan State University, E. Lansing, USA.
(2) F.A. Janssens Laboratory of Genetics, K.U.Leuven, Belgium

Azospirillum brasilense belongs to a group of plant growth promoting bacteria, capable of fixing nitrogen under micro-aserobic conditions. The regulation of expression of the *A. brasilense* nitrogen fixation (*nif*) genes is less well understood than in other diazotrophs. NtrA dependent promoters have been characterized for *A. brasilense* and a regulatory gene *nifA* has been isolated (7,8). The *ntrA* gene (encoding sigma 54) of *A. brasilense* Sp7 was isolated by using conserved *ntrA* primers and PCR, and its nucleotide sequence was determined. The putative NtrA protein shares high degree of homology with other members of the sigma 54 family. Two additional open reading frames were found in the *ntrA* region, with significant similarity to equivalent regions surrounding the *ntrA* locus in other bacteria. An *ntrA*::Tn5 mutant of strain *A. brasilense* Sp7 was found to be defective in nitrogen fixation and nitrate assimilation, but C4 dicarboxylic acid uptake, shown to be NtrA controlled in other nitrogen fixing bacteria, was not affected. As found for *Pseudomonas* sp (5,10), *Caulobacter* (9) and *Campylobacter* (1), the *ntrA* mutant was non-motile, suggesting a role of NtrA in flagellar biosynthesis. *A. brasilense* mutants in *ntrA*, *ntrB* (4) and *ntrC* (6) were unable to take up [C14]methylammonium. This indicates that the *ntr* system may regulate the activity of an ammonium carrier. However, *ntr* mutants are able to grow on low ammonium concentrations. These seemingly contradictory observations can be explained by assuming the existense of (at least) 2 types of ammonium carriers in *A. brasilense* with only the one that is regulated by the ntr system, transporting [C14]methylammonium. This situation also occurs in *Rhodobacter sphaeroides, R. capsulata* (3) and *Anacystis nidulans* (2).

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CLONING, SEQUENCING, AND PHENOTYPIC ANALYSIS OF *laf1*, ENCODING THE FLAGELLIN OF THE LATERAL FLAGELLA OF *Azospirillum brasilense* Sp7

MOENS, S., MICHIELS, K.[1], KEIJERS, V., VAN LEUVEN, F.[2]
AND VANDERLEYDEN, J.[1]
[1] F. A. Janssens Laboratory of Genetics, Willem de Croylaan 42, B-3001 Heverlee, Belgium
[2] Center of Human Genetics, Campus Gasthuisberg, B-3000 Leuven, Belgium

Azospirillum brasilense can display a single polar flagellum and several lateral flagella (Tarrand *et al.*, 1978). The *Azospirillum brasilense* Sp7 gene *laf1*, encoding the flagellin of the lateral flagella, was isolated and sequenced. The derived protein sequence is extensively similar with the flagellins of *Rhizobium meliloti*, *Agrobacterium tumefaciens*, *Bartonella bacilliformis* and *Caulobacter crescentus*. Amino acid alignment shows that the flagellins of these bacteria are clustered and clearly different from other known flagellins. A *laf1* mutant, FAJ0201, was constructed by replacing an internal part of the *laf1* gene by a kanamycin resistance encoding gene cassette. The mutant is devoid of lateral flagella but still forms the polar flagellum. This phenotype is further characterized by the abolishment of the capacity to swarm on a semi-soli surface and to spread from a stab inoculation in semi-solid medium. FAJ0201 shows a normal wheat root colonization pattern in the initial stage of plant root interaction.

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GLUTAMATE SYNTHASE: PROPERTIES OF THE *AZOSPIRILLUM BRASILENSE* ENZYME AND OF ITS SUBUNITS EXPRESSED SEPARATELY IN *E. COLI*

VANONI, M.A., VERZOTTI, E., CURTI, B. AND ZANETTI, G.
Dipartimento di Fisiologia e Biochimica Generali, Sezione di Chimica Biologica, Universita' degli Studi di Milano, Via Celoria 26, 20133 Milano, Italy.

Glutamate synthase (GltS) is a key enzyme in cellular nitrogen metabolism. The GltS from *Azospirillum brasilense* has been purified in our laboratory. It is a complex iron-sulfur flavoprotein which contains 1 FAD, 1 FMN and three distinct Fe/S centers per αβ protomer (α subunit, ca. 140 kDa; βsubunit, ca. 56 kDa). Kinetic, mechanistic and spectroscopic studies led us to propose a model for the active center of the enzyme: during catalysis NADPH reduces the enzyme flavin at Site 1 (Flavin 1); the electrons flow through Center I (the 3Fe/4S center of GltS) and Center II (presumably a 4Fe/4S Center) to the flavin at Site 2 (Flavin 2) where reduction of the imino acid formed on addition of ammonia (from glutamine) to 2-oxoglutarate takes place. The GltS protomer also contains another Fe/S center (Center III, presumably also a 4Fe/4S center) whose potential appears too low to be involved in the electron transfer process (Vanoni et al., 1992). The genes encoding the enzyme subunits have been cloned, sequenced, and the primary structure of the enzyme has been analyzed (Pelanda et al., 1993, Vanoni et al., 1994). Engineering of the *Azospirillum glt* genes has allowed us to express the individual subunits in *E. coli*. The recent characterization of the functional and structural properties of the isolated GltS subunits showed that, contrary to expectations only one flavin (FMN) is bound to the GltS α subunit, while FAD (which exhibits properties previously assigned to Flavin 1 of GltS) is on the ß subunit. The latter binds NADPH which reduces bound FAD at a rate consistent with GltS turnover. Furthermore, the isolated β subunit exhibits NADPH-dependent diaphorase activities but no glutamate synthase or glutamate dehydrogenase activities in either direction. Taken together these results are in agreement with previous data from our laboratory, but against the early hypothesis that GltS evolutionary derives from the association of an ancestral glutamate dehydrogenase (the ß subunit) with an amidotransferase (the α subunit). Rather, from the present data it appears that, in GltS, the ß subunit acts as a NADPH:acceptor oxidoreductase which provides reducing equivalents for reduction of the imino acid bound on the α subunit.

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INVOLVEMENT OF THE P_{II} REGULATORY PROTEIN OF *Rhizobium meliloti* IN NITROGEN FIXING SYMBIOSIS

ARCONDEGUY, T., HUEZ, I., GANGNEUX, C., TRUCHET, G. AND KAHN, D.
Laboratoire de Biologie Moleculaire des Relations Plantes-Microorganismes CNRS-INRA, BP27, 31326 Castanet-Tolosan Cedex, France

During the differentiation of *Rhizobium* bacteroids, nitrogen metabolism switches from ammonia assimilation to ammonia export. In principle this switch may involve either genetic or metabolic regulation of glutamine synthetase (GS) activity, both of which are affected by the P_{II} regulatory protein.

To investigate the role of P_{II} in nitrogen fixing symbiosis, we constructed an in-frame deletion within the P_{II} gene *glnB*. The resulting mutant of *R.meliloti* exhibits a pleiotropic phenotype. Surprisingly, it expresses no GSII, it exhibits delayed nodulation, and although the resulting nodules reduce acetylene, alfalfa plants appear nitrogen starved and chlorotic.

One well known target of P_{II} in enteric bacteria is adenylylation of GS. To assess the role of GSI agdenylylation in symbiosis, we constructed a non adenylylatable GSI mutant by oligonucleotide directed mutagenesis. We anticipated that such a mutant might perturb the flux of ammonia from bacteroids to the host plant. Contrary to this expectation, this mutant exhibited an essentially wild-type $Nod^{+}Fix^{+}$ phenotype. Thus, the effect of the *R.meliloti glnB* mutation on symbiosis must be due to other effects which are being characterized.

ASSOCIATIVE INTERACTIONS OF *AGROBACTERIUM RADIOBACTER* 5D-1 WITH MONO- AND DICOTYLEDONOUS PLANTS

CHUMAKOV M.I., SOLOVOVA G.K., VELIKOV V.A., GORBAN V.V., KRIVOPALOV YU.V., DYKMAN L.A., BOGATIREV V.A., AND KENNEDY, C. (1)
Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, Saratov 410015, Russia
(1) Dept.Plant Pathology, University of Arizona, Tucson 85721,USA

Agrobacterium radiobacter strain 5D-1 was isolated in 1986 from wheat roots after surface sterilization and reisolated from wheat roots in 1994 as *A.radiobacter* 5D-2. Both strains showed similar culture characteristics and patterns of *EcoRI*, *HindIII* DNA restriction fragments. Strain 5D-1 exhibited a number of associative traits. a) It can colonize and attach to mono- and dicotyledonous plants. Strain 5D-1 was able to attach to wheat and rice roots in higher numbers than did pathogenic agrobacteria. The mode of attachment of non-pathogenic agrobacteria to monocotyledonous plants was similar to attachment of pathogenic agrobacteria to dicotyledonous plants (Solovova et al.,1995). b) It produces phytohormones (IAA, cytokinin). c) It has positive growth promotion effects on wheat, tomato and sunflower. Strain 5D-1 inoculation effect could be explained by competition with other bacteria for the attachment site on the plants roots and high-level phytohormone production (up to 9-25 mg/L medium). We did not observe any strong repressive effect of strain 5D-1 on *Agrobacterium tumefaciens*, but recorded a positive effect of *A.radiobacter* preinoculation on tomato stems before *A.tumefaciens* infection. *A.radiobacter* 5D-1 carrying the *Sym*-plasmid from *Rhizobium galegae* can produce nodules on alfalfa roots. We recorded low acetylene-reduction activity of *A.radiobacter* strain 5D-1 in pure culture in free living conditions (3 nm C_2H_4/ml medium per day). In association with sterile wheat seedlings, a higher level of acetylene reduction was found (200-1300 C2H4 nm/ml medium per plant per day). We did not observed strong positive hybridization signal from total DNA with *nifH* from *R.meliloti* or *nifHDK* from *A.brasilense*. Thus, no evidence was found for *nif* genes in *A.radiobacter* 5D-1 or 5D-2.

A.radiobacter 5D-1 was able to attach to wheat roots (Chumakov et al.,1992) and to produce fibril-like structures (FLS) (Solovova et al.,1995). Using mutant strains, we hypothesize that flagella and surface polysaccharides of A.radiobacter 5D-1 are involved in the attachment process to wheat roots.

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AGROBACTERIUM RADIOBACTER - A MODEL FOR STUDYING OF PLANT-MICROBE INTERACTION AND NITROGEN METABOLISM IN ASSOCIATION.

KAMENEVA S.., MURONETS E., BELAVINA N., RUSLJAKOVA M.Department of Genetics, Biology Faculty, Moscow State University, Moscow,119899, Russia.

The saprophytic *Agrobacterium radiobacter* 5D-1 strain is a suitable model organism for investigation of the genetic control and molecular mechanisms of plant-microbe interaction and nitrogen metabolism in association. This strain has been isolated from wheat roots.(M.Chumakov et al., 1992).It have been shown to increase acetylene reduction activity in association with plants, to produce indoleacetic acid and to form the fibrils for attachment to the roots surface.

Methods for conjugative transfer of large transmissible plasmids, vectors, transposons and chromosomal genes were elaborated. Donor clones with polar chromosome transfer were obtained. The plasmid and chromosomal genes transformation into 5D-1 strain and its derivatives was performed. The first circular genetic map of *A.radiobacter* chromosome has been constructed..

The Tn5-mutants of 5D-1 strain, altered in ability to interact with plants (Cal) and in nitrogen metabolism (Nm and Nr) have been isolated. Cal mutants were found to be deficient in the formation of fibrils for attachment to the rape root surface; they have the same acetylene reduction activity as wild type in pure culture and decrease one in the association with rape. Nm-mutants were able to use glutamine and glutamate or ammonium sulfate as sole source of nitrogen compound; Nr- used glutamine or ammonium sulfate only. Nm and Nr mutants have the different levels of acetylene reduction activity (increased or decreased)in pure culture as well as in association in rape. The mutants with increased acetylene reduction activity in association or enhanced indoleacetic acid production stimulate the plant growth and productivity.

The mutants altered in nitrogen metabolism were localized on the genetical map of *A.radiobacter*. We are currently use these mutants for cloning *A.radiobacter* genes required for association with plants.

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CHARACTERIZATION OF NITROGEN FIXATION IN MAIZE ROOT ASSOCIATED KLEBSIELLA PLANTICOLA 19-1

LI J-D, JIN H-F, WANG J-W, DONG Y-M, LI Y-X
Institute of Botany, Academia Sinica, Beijing 100044 China

A strain K19-1 was an associative nitrogen fixation bacterium isolated from maize roots grown in Shanshan area, Xinjian Uygu- Autonomous region. It was identified as Klebsiella planticola. K19-1 could grown in N_2-free medium at 40°C and reduced acetylene and $^{15}N_2$ at 40°C. The nifH promoter::lacZ fusion was expressed in K19 at 40°C, NH_4^+ did not inhibit nitrogenase activity but repressed the expression of a nifH::lacZ fusion in K19-1. K19-1 could fix nitrogen aneanobically but nitrogenase activity was still not inhibited till O_2 concentration was increased to 4%. The expression of nifH::lacZ fusion in K19-1 was not repressed by O_2. (Li et al 1994)

A large plasmid (about 300kb) in K19-1 was identified from lysis of bacteria in an agarose gel and using a technique of electrical field overturn. The presence of nifHDK-like gene and localization of a nifHDK-like gene in the large plasmid were confirmed by a southern hybridization with a ^{32}P-labelled nifHDK probe. (Li et al 1994) By using K.pneumoniae nif'BAL' gene fragment as a probe for molecular hybridization. The presence of a nifA-like gene in a large plasmid in K19-1 was identified. A DNA fragment about 5.4Kb containing the nifA-like gene was cloned. The restriction map and the location of nifA-like gene in the 5.4kb fragment were analyzed. The plasmid pLJ80 contained 5.4kb fragment was transfered to the $nifA^-$mutant (UN1770) and transformant with nitrogenase activity was recovered. (Jin et al 1995)

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IDENTIFICATION OF *NIFA* AND *NTRBC* GENES IN *ACETOBACTER DIAZOTROPHICUS*

MELETZUS, D.[3], PERO, R.[1], KENNEDY, C.[1], TEIXEIRA, K.[2] AND BALDANI, I.[2]
[1] Department of Plant Pathology, College of Agriculture, The University of Arizona, Tucson, AZ 85721, USA
[2] EMBRAPA, Centro Nacional de Pesquisa de Biologia do Solo, Seropedica, 23851 Rio de Janeiro, Brazil
[3] Department of Microbiology and Genetechnology, Faculty of Biology, University of Bielefeld, 33501 Bielefeld. Germany

Acetobacter diazotrophicus is a diazotrophic endophyte isolated from roots, stems and leaves of sugarcane. A possible role in contribution of fixed nitrogen to the host has been concluded from nitrogen-15 balancing experiments as well as growth of sugarcane on N-deficient soils. In a genetic attempt we are currently isolating genes known to be involved in nitrogen fixation. Mutants will then be used to evaluate the interaction of *A. diazotrophicus* with its host plant.

A wide host range genomic library of *Acetobacter diazotrophicus* PAL5 was prepared and transferred to several recipient strains of *Azotobacter vinelandii* or *E. coli*. Cosmids complementing a *nifA* mutation in *A. vinelandii* and *ntrC* mutations in either *A. vinelandii* or *E. coli* were isolated. Interestingly, cosmids carrying the *A. diazotrophicus nifA* gene were able to complement *A. vinelandii ntrC* mutants. The *nifA* gene was mapped physically by introduction of deletions into cosmid pAD101. Initial sequencing data reveal the localization of a *nifB*-like gene downstream of *nifA*.

Determination of the nucleotide sequence of the *ntrBC* region on cosmid pAD201 revealed the existence of an additional open reading highly homologous to *nifR3* genes. Therefore, as in *Azospirillum brasilense* and *Rhodobacter capsulatus*, the *ntrBC* genes in *A. diazotrophicus* are organized in a *nifR3-ntrBC* gene cluster.

INDOLEACETIC ACID PRODUCTION IN *AGROBACTERIUM RADIOBACTER*.

MURONETS, E., BELAVINA,N., MITRONOVA,T., KAMENEVA, S.
Dept. of Genetics, Biology Faculty, Moscow State University,
119899 Moscow, Russia.

The associative soil bacterium *Agrobacterium radiobacter* 5D-1 has been isolated from the surface sterilized wheat roots (Chumakov et.al.,1992). The capacity of this strain to promote plant growth is presumed to depend on several bacterial properties. One of these factors could well be the production of phytohormones, e.g. indole-3-acetic acid (IAA). It was shown that IAA-production in wild type, detecting by Salcowski reaction, depends on tryptophan maintenance in culture medium and correlates with increasing of tryptophan concentration. The highest level of IAA synthesis is occurred after 72 h incubation in mineral medium with glucose or mannitol. IAA synthesis is suppressed by ammonium sulfate (10 mM) or glutamine (2, 10 mM). The inhibition of IAA synthesis by ammonium sulfate and glutamine was observed not only in wild type but also mutants with altered nitrogen metabolism (Nr-224, Nm-17). The inhibitory action to mutants became apparent to a greater extent than wild type. The pathogenic *Agrobacterium tumefaciens* produces IAA via the indole-3-acetamide pathway, for which the genes are Ti-plasmid borne. It does possess also the indole-3-pyruvic acid pathway, which is chromosomally encoded (Costacurta, Vanderleyden, 1995). In nonpathogenic *A.radiobacter* 5D-1 the pTi is absent and IAA production level is about 25% of *A.tumefaciens* C58. The 260 MD pVM plasmid is present in 5D-1, but it does not control the IAA synthesis (Muronets et al., 1994). The strains with quantitative and qualitative changes in IAA production were revealed among the mutants altered in nitrogen metabolism (Nr,Nm) and formation of calcofluor-binding structures (Cal). Cal-3,-24 mutants possess the increased IAA production level. Nm-mutants differ from wild type slightly, Nr possess 30-40% IAA production level only. The mutants with enhanced IAA synthesis stimulate the early stages of rape and tomato plant growth. However the stimulating influence of many mutants to the plants is more complex.

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DELETION ANALYSIS OF THE PROMOTER REGION OF THE *NIFA* GENE FROM *HERBASPIRILLUM SEROPEDICAE*.

SOUZA, E.M. (1), MACHADO, H.B. (2) AND YATES, M.G. (3).
(1) Dep. de Bioquímica, UFPR, C.P. 19046, 81531-990 Curitiba - PR, Brazil.
(2) Dep. Farmacologia, UFPR, 81531-990, Curitiba - PR, Brazil.
(3) NFL-AFRC, University of Sussex, BN1 9RQ, Brighton, England.

Herbaspirillum seropedicae is an endophytic diazotroph found associated with gramineae such as sugar-cane and sorghum. The *nifA* gene of this organism was cloned and sequenced (Souza et al., 1991a,b). In order to study the *nifA* gene expression several deletions were constructed in the promoter region and cloned into the transcriptional *lacZ* fusion vector pMP220 or pPW452. Expression of the *nifA* gene was repressed (80%) by NH_4Cl, but not by air. Similar levels of expression were obtained in the wild-type *H. seropedicae* and in the *nifA* mutant Smr54, suggesting that the NifA protein is not involved in *nifA* expression in this organism. Deletion of sequences upstream from base 264, including a half NtrC binding-site, decreased the promoter activity under N_2-fixing conditions to levels only slightly higher than those under repressive conditions. In *E. coli,* the expression of the *H. seropedicae nifA* gene was found to be dependent on RpoN and activated by the NtrC or NifA proteins. Further deletions of 179 and 190 bp caused an increase in the β-galactosidase activity under N_2-fixing conditions, which was repressed by either ammonium ions or air in the wild-type strain , while in the *nifA* mutant there was no response to the absence of fixed nitrogen and low oxygen.

Our results indicate that the *nifA* gene from *H. seropedicae* exhibit a low level of constitutive expression, possibly from a uptream non-identified promoter. Under low ammonia conditions the NtrC protein activates the expression from a -24/-12 promoter located at position 652-665. The NifA binding site apparently is not functional when the promoter region is intact, becoming active once the 5' sequences are removed.

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THE N-TERMINUS OF THE NIFA PROTEIN OF *HERBASPIRILLUM SEROPEDICAE* IS PROBABLY INVOLVED IN SENSING OF AMMONIA

SOUZA, E.M. (1), PEDROSA, F.O. (1), MACHADO, H.B.(2), DRUMMOND, M. (3) AND YATES, M.G. (3).
(1) Dep. Bioquímica, UFPR, C.P. 19046, 81531-990, Curitiba - PR, Brazil.
(2) Dep. de Farmacologia, UFPR, 81531-990, Curitiba - PR, Brazil.
(3) Nitrogen Fixation Laboratory, AFRC, BN1 9RQ, Brighton, England.

The NifA protein is the specific activator of *nif* genes expression. Three domains have been found in the NifA protein, namely the N-terminal, Central and C-terminal domains (Drummond et al.,1986). The roles of the Central and C-terminal domains have been established: interaction with the transcriptional apparatus and DNA-binding, respectively. The function of the N-terminal domain, however, remains to be determined.

The protein NifA of *Herbaspirillum seropedicae* was found to be unable to activate expression from the *nifH* promoter of *Klebsiella pneumoniae* in an *Escherichia coli* background. A deletion of the first 606 bp from the 5' region of the *nifA* gene was fused to the *lacZ* gene led to a truncated protein capable of activating *K. pneumoniae nifH* promoter in *E. coli* under anaerobic conditions. The truncated NifA protein was inactive under air and in the absence of iron, but was not affected by the presence of ammonia. Manganese, nickel or cobalt did not substitute for iron. In *Azospirillum brasilense* the native NifA protein of *H. seropedicae* failed to activate expression of *nifH* under high NH_4Cl concentration. The truncated NifA protein, however, was active in the presence of ammonia. Both truncated and intact NifA were inactive under air and in the absence of iron in *A. brasilense*.
These results indicate that the activity of the NifA protein of *H. seropedicae* is inhibited by ammonium ions and oxygen, and is dependent on iron. Furthermore its N-terminus appears to be involved in the regulation of NifA activity by ammonium ions.

Drummond et al. (1986) EMBO J. 5, 441-447.

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UTILIZATION AND REMOVAL OF NITROGEN OXIDES IN RHIZOBIA

TOFFANIN A. (2), RITZERFELD B. (1), SHAPLEIGH J.P. (2), PAYNE W.J. (3) and CASELLA S. (1)
(1) Dip. Biotecnologie Agrarie, Università di Padova, 35131 Padova, Italy
(2) Dep. of Microbiology, Cornell Univ., Ithaca, NY 14853-8101, USA
(3) Dep. of Microbiology, Univ. of Georgia, Athens, GA 30602-2605, USA

Several species of rhizobia are able to reduce nitrogen oxides to more reduced states ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$). Although some strains behave like true denitrifiers by coupling NO_X reduction to energy conservation (J.P. Shapleigh, W.J. Payne, 1985; B. Bhandari et al., 1984), some others seem to reduce nitrogen oxides, especially nitrite, without any production of electrochemical gradient (S. Casella et al., 1986). Within the latter group the expression of the reductases occurs only under low oxygen conditions suggesting a protective role for a nitrite sensitive component (i.e. nitrogenase)(S. Casella et al., 1988). In the present work we describe a strain of *Bradyrhizobium japonicum* that, when grown anaerobically, produces free NO during nitrite reduction to N_2O. This is similar to most other denitrifiers. However, when this strain is grown with a 5% initial oxygen concentration, no free NO is detectable during nitrite reduction, at least as assayed in the presence of the uncoupler CCCP and cytochrome *c* (I. Kucera, 1989). Moreover, no N_2O reduction occurs. This apparent lack of free NO is similar to what is observed in a fast-growing strain of *Rhizobium 'hedysari'*. Under low oxygen this strain, which cannot grow anaerobically by respiration of nitrogen oxides, produces no detectable NO, at least with the method mentioned above, during nitrite reduction to N_2O. These two modes of nitrite reduction suggested that there may be more than one type of nitrite reductase. Since the *R. 'hedysari'* strain appears to have only the non-denitrifying type of reductase we started working to characterize this enzyme. The nitrite reductases in both rhizobia were completely inhibited by the copper-chelator diethyldithiocarbamate (DDC), suggesting a Cu-type Nir. This was confirmed by Southern blot using a heterologous DNA probe derived from the gene encoding the copper Nir from *Rhodobacter sphaeroides* 2.4.3. (Shapleigh, unpublished). The patterns of hybridized bands indicate that all the strains tested, except *R. 'hedysari'* CC1335, contain the copper-Nir structural gene. About 70% of *nir* from *R. 'hedysari'* HCNT1 has been cloned and sequenced. It is closely related to other Nir. For example, it has 81% identity with Nir from *A. cycloclastes* . Southern blot using a probe derived from the gene encoding NO reductase from *R. sphaeroides* (Shapleigh, unpublished) also hybridized to chromosomal DNA from HCNT1 as well as other rhizobia.

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SECTION 4

SIGNALLING PROCESSES IN PLANT-MICROBE INTERACTIONS

SIGNALLING PROCESSES IN PLANT-MICROBE INTERACTIONS: INTRODUCTORY REMARKS.

SPRENT, J.I.
Department of Biological Sciences, University of Dundee, Dundee DD1 4HN, UK

The papers cover the three main aspects of signalling, namely:

1.Production of flavonoids and related compounds by host roots, leading to...
2.Production of extracellular compounds by rhizobia, leading to...
3.Nodule initiation.

All of these processes are complex as are their interactions. We shall first take them in turn and then attempt a synthesis.

1.Production of flavonoids and other compounds by plants

Phillips and co-workers have identified 14 flavonoids and 2 betaines in exudates of alfalfa roots. They examined the ability of these compounds to induce nod gene transcription. They also looked at their effects on a variety of mutants of *Rhizobium meliloti* (all Nod+) and *Agrobacterium tumefaciens* (all Vir+). Novel loci were identified, responding to inducers and affecting the ability of the mutants to colonize roots in competition with parent strains.

The work of Coopers group shows that rhizobia can metabolise nod gene inducing flavonoids, producing 3 groups of metabolites which may be active in bacterial-plant communication.

2.Production of extracellular compounds by rhizobia

(a)Lipo-chitin oligosaccharides (LCO). There are 3 distinct parts to these molecules; first the backbone consisting a tetramer, pentamer or hexamer of N-acetyl glucosamine. Although coded by the common nod genes (nod ABC) it possibly has a role in specificity (Debelle et al): second, the highly unsaturated, hydrophobic, fatty acid moiety. This may vary in structure (Spaink): third substitutions on the reducing end of the backbone, which may vary from simple units such as acetyl to more complex ones such as 2-0-methylfucosyl (Stacey).

Rather than a simple physical fit (lock and key) between LCOs and receptor molecules on the plant surface (e.g. lectins), the situation appears to be more complex, with all three parts of the LCO being involved in the exchange of signals between host and

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bacteria. For example bradyrhizobia nodulating soybeans produce both tetrameric and pentameric LCOs (Stacey). Fucosylation of the terminal reducing end of the sugar is necessary for activity of the pentamer, but inactivates the tetramer.

(b)Others. Nod O is a protein secreted by *R. leguminosarum bv. vicia* (Downie et al.). This protein is able to bind Ca2+ and under patch-clamping forms pores in membranes. It apparently has a role complementary to that of LCOs.

A range of extracellular factors, unrelated to their chromosomal lineages, is produced by rhizobia nodulating common beans (*Phaseolus vulgaris*) (Martinez et al.). It is suggested that these may respond to strong selective pressures enabling the rhizobia to keep a step ahead of plant defence reactions.

Butanol extracts of bradyrhizobial cultures may have effects quite separate from LCOs, for example inducing ENOD2 (Stacey).

3.Nodule initiation

This is reviewed by Hirsch, but also touched on by Spaink and others. Spaink points out that one of the effects of nod factors is to cause de-differentiation of cortical cells of roots, and stimulation of cell division. Auxin transport inhibitors (ATIs) can produce similar responses and also elicit expression of ENOD2 (see above). Cytokinins or ATIs can elicit the expression of ENOD2, 12 and 40, mimicking some of the effects of LCOs and butanol of extracts of bradyrhizobia. Endogenous plant hormones thus also have a role to play in nodule initiation.

Synthesis

Compounds exuded from plant roots have multiple effects on rhizobia and other soil microorganisms. Those from alfalfa alone affect nod induction and root colonization (both by rhizobia and agrobacteria). In the field, the situation is likely to be complicated further by products of roots of other plants. Rhizobia may metabolise compounds in plant root exudates, giving a range of products, any of which might take part in the signalling processes and contribute to specificity. Again, in a field situation an array of compounds is likely to be present which may affect nodulation and possibly interact with bacterial pathogens. One way in which the rhizobia may cope with the complexity of soil chemistry is suggested by work on *Bradyrhizobium japonicum* which has two independent systems, each of which may be induced by plant-produced isoflavones, leading to production of nod factors (Stacey).

Nod factors, since first isolated and characterised have tended to be associated with host specificity, particularly with respect to the nature of the residue attached to the reducing end of the backbone. Papers in this session have indicated that all 3 parts of LCOs are involved in specificity. Further, successful production of nodules probably requires other compounds to permit Ca^{2+} signalling and repress the host plants' defence responses, and unidentified butanol-soluble substances to induce specific nodulins.

Whilst nodule initiation is affected by factors produced by rhizobia as well as endogenous phytohormones, it is also influenced by other processes. Various soil microorganisms produce phytohormones affecting root growth, root hair formation and, almost certainly, nodulation. Various proteins produced by both roots and nodules may or may not be influenced by nod factors and/or phytohormones (Hirsch). Lectins are a particularly interesting class of such protein. For many years they have had a rather equivocal place in the nodule literature, but it is now becoming clear that they may have a necessary but not sufficient role to play in explaining host recognition and possibly other aspects of nodulation, such as rhizobial adsorption.

Although this session has shown how complex the signalling processes between plants and rhizobia are, even with single genotypes and strains, a more complete picture is emerging. At the same time we can begin to appreciate that, in the soil, the presence of other plants and other microorganisms can complicate matters, resulting in a spectrum of responses from enhanced to reduced nodulation.

DETERMINANTS OF NODULATION EFFICIENCY IN THE SYMBIOSIS BETWEEN *PISUM SATIVUM* AND *RHIZOBIUM LEGUMINOSARUM*

DOWNIE, J.A.(1), SUTTON, J.M.(1), DEAN, G.(1), DAVIES, A.E.(1), FINNIE, C.(1),GHELANI, S.(1), LEA, E.J.A.(2), WILSON, K.E.(1) AND FIRMIN, J.(1)
(1)John Innes Centre and (2)University of East Anglia, Norwich Research Park,Colney, Norwich, NR4 7UH, UK.
Tel. 44(1603)52571, Fax.44(1603)456844

Abstract

Rhizobium leguminosarum **biovar *viciae* induces the formation of nitrogen-fixing nodules on the roots of legumes such as pea and vetch. This is dependent on the *R.l. viciae nod* gene products, several of which are involved in the synthesis of lipo-chito-oligosaccharide signalling molecules (Nod factors). In a specific line of *Pisum sativum* (pea), nodulation is strongly inhibited by *R. l. viciae* strains that overproduce these Nod factors suggesting that the amount of Nod factor made can be crucial for optimal nodulation. Some of the *R.l. viciae nod* gene products are not involved in the synthesis of these Nod factors. The possible roles of one of these (NodO) is assessed with regard to its biochemical role and how this secreted protein acts to confer specificity in nodulation of pea and vetch.**

Keywords: *Rhizobium*, nodulation, Nod metabolite, protein secretion, specificity

1. Introduction

Rhizobium leguminosarum biovar *viciae* interacts with the roots of species of *Pisum*, *Vicia*, *Lens* and *Lathryus* to induce the formation of nitrogen-fixing nodules. This is initiated by signal exchange between the two symbionts. Flavonoids secreted from legume roots are recognised by the bacteria via the *nodD* gene product which belongs to the family of LysR-type regulators (Schlaman *et al.*, 1992). In the presence of the appropriate flavonoid, such as hesperetin or eriodictyol, (Firmin *et al.*, 1986; Zaat *et al.*,1987) twelve *Nod* genes in four operons *nodABCIJ*, *nodFEL*, *nodMNT* and *nodO* are induced (Downie, 1991). The *nodABCFELMN* genes all appear to be involved in the biosynthesis of lipo-chito-oligosaccharide nodulation signalling molecules that are made up of four or five β1-4-linked N-acetyl glucosamine residues in which the terminal non-reducing residue carries an N-linked acyl group and an O-linked acetyl group (Spaink *et al.*, 1991; Downie, 1994). As reviewed recently (Carlson *et al.*, 1994; Downie, 1994) it is now apparent that: *nodM* encodes a glucosamine synthase involved in providing glucosamine precursors; *nodC* encodes a glucosaminyl transferase that forms the oligo-glucosamine backbone; *nodB*

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encodes a deacetylase that removes the N-linked acetyl residue from the terminal glucosamine; *nodA* encodes a protein involved in the acylation of the amino group exposed by the action of NodB; *nodF* and *nodE* encode proteins involved in the biosynthesis of the unusual C18:4 acyl group attached to the amino group; and *nodL* encodes an O-acetyl transferase that O-acetylates the acetylated glucosamine residue at the non-reducing end. Although a role for the *nodN* gene product has not been identified, Baev *et al.* (1991) showed that (as had been observed for *nodM*) mutation of *nodN* in *R. meliloti* reduced the amount of Nod factor made, suggesting a possible role in the synthesis of precursors required for Nod factor biogenesis. Thus *R.l. viciae* has a group of genes dedicated to the synthesis of Nod factors.

It appears likely that the biosynthesis of these Nod factors occurs in the cytoplasm since none of the enzymes involved in the biosynthesis appear to be targeted to the periplasm. Thus there is most likely to be a requirement for export of the Nod factors. Evans and Downie (1986) noted that the *nodI* gene product belongs to a group of proteins involved in ATP dependent transport and that NodJ has the characteristics of an integral membrane protein. Vazquez *et al.* (1993) suggested a possible role for NodI and NodJ in Nod factor secretion and McKay and Djordjevic (1993) demonstrated that mutation of these genes in *R.l. trifoli* reduced the level of Nod factor secretion. Rivilla *et al.* (1995) showed that NodT is an outer membrane protein and suggested that it might function together with NodI and NodJ to form a complex that might translocate Nod factors directly across both inner and outer membranes, thus potentially delivering Nod factors to the plant.

It appears that the *nodO* gene product is not involved in the biosynthesis or secretion of Nod factors. In fact like Nod factors, it too is secreted and thus is likely to interact with plant cells. Mutation of *nodO* does not have much effect on nodulation of peas or vetch. However in the absence of *nodE* (when nodulation of peas is about 50% of normal) *nodO* is required for nodulation (Downie and Surin, 1990; Economou *et al.*, 1995). In this work we address the possible role of NodO in determining efficiency of nodulation. In addition we analyse how the amounts of Nod factor made can influence the efficiency of nodulation in specific circumstances.

2. Inhibition of nodulation by strains overproducing Nod factors

Cultivar Afghanistan peas are not nodulated by most *R. l. viciae* strains isolated from western soils, but can be infected by other isolates such as strain TOM. This strain contains an additional nodulation gene (*nodX*), the product of which O-acetylates the reducing glucosamine residue on the Nod factor (Firmin *et al.*, 1993).

In co-inoculation experiments, some of the strains unable to nodulate cv Afghanistan peas inhibit nodulation by strain TOM and this inhibition is dependent on those genes involved in Nod factor biosynthesis (Dowling *et al.*, 1989; Firmin *et al.*, 1993). The observations that, in addition to the NodX-acetylated Nod factor, *R. l. viciae* strain TOM makes the same range of Nod factors as western strains (Firmin *et al.*, 1993) suggested that the nodulation inhibition was not simply caused by production of the inappropriate Nod factors (i.e. the range of normal Nod factors made by strain TOM does not inhibit its own ability to nodulate cv Afghanistan).

A characteristic shared by those strains that inhibit nodulation of cv Afghanistan by strain TOM is that they produce high levels of Nod factor. By cloning the *nod* gene region from strain TOM on a multicopy plasmid (pIJ1095) and transferring it to the Sym$^-$ strain of *R. l. viciae* (strain B151) we have made a strain that produces the same range of Nod factors as strain TOM but at a level equivalent to that produced by nodulation-inhibiting strains. This strain (B151/pIJ1095) nodulates cv Afghanistan peas very poorly, whereas it nodulates western varieties of pea normally. In co-inoculation experiments, strain B151/pIJ1095 strongly inhibits nodulation of cv Afghanistan peas by strain TOM even though both strains make the same range of Nod factors.

In the light of the above observations it appeared likely that the poor nodulation of cv Afghanistan peas by B151/pIJ1095 might be due to this strain inhibiting its own nodulation because it produced too much of the Nod factors. We identified a derivative of B155/pIJ1095 containing Tn*5* in pIJ1095 and this strain was partially restored for nodulation on cv Afghanistan peas. DNA sequencing revealed that the Tn*5* was inserted in *nodD*. Analysis of the growth medium supernatant from this strain showed that low levels of Nod factors were made. Therefore we conclude that when cv Afghanistan peas are exposed to high levels of Nod factors, nodulation is inhibited.

3. NodO is a secreted protein

The DNA sequence of *nodO* (de Maagd *et al.*, 1989; Economou *et al.*,1995) showed that it encodes a protein with marked similarity to a group of proteins including haemolysins, cyclolysins, leukotoxins and several metalloproteinases. These proteins all have in common a Ca^{2+}-binding domain that is made up of several tandem nonapeptide repeats. The crystal structure of one such protein has been solved and the nonapeptide repeats form a novel β-roll structure (Baumann *et al.*, 1993). The protein sequence homology between NodO and the other proteins was essentially limited to that domain. This group of proteins also has in common the property that they are secreted by a mechanism that does not involve an N-terminal transit peptide and the general secretion pathway. Instead, they appear to have a C-terminal secretion signal which is recognised by a specialised secretion system. For haemolysin A, secretion in *E. coli* requires the HlyB HlyD and TolC proteins; NodO secretion by *E. coli* is also dependent on the HlyBD-TolC system (Scheu *et al.*, 1992). To determine if NodO secretion requires a C-terminal signal, a series of deleted forms of NodO was constructed. Deletion of the C-terminal 24 amino acids (Fig. 1) resulted in the formation of a stable protein that could be detected in the cytoplasm of *R.l. viciae* but which could only be secreted at very low levels ($\approx$5% of normal). A series of internal deletions that retained the C-terminal 24 residues of NodO (Fig. 1) permitted normal secretion indicating that this domain may be sufficient to allow the *R.l. viciae* NodO secretion system to recognise the deleted derivatives of NodO.

Scheu *et al.* (1992) showed that although the *nodO* gene could not be detected in several other rhizobia, they all had the ability to secrete NodO protein if the *R.l. viciae nodO* gene was transformed into them. Crank *et al.* (1994) demonstrated that rhizobial strains

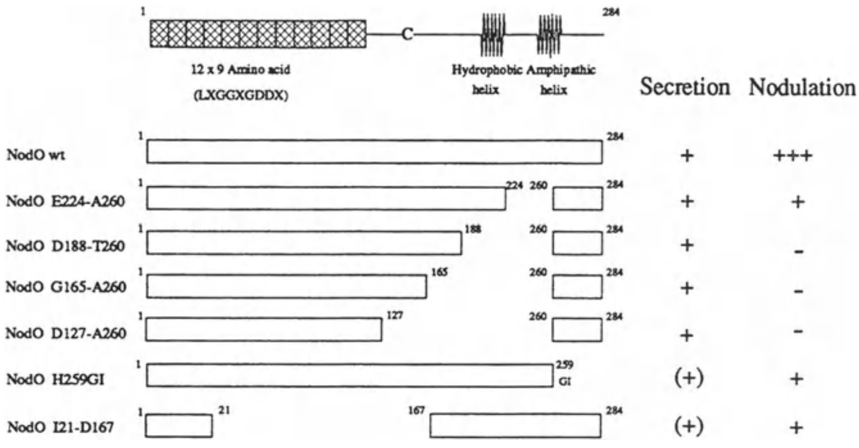
1
284
C
12 x 9 Amino acid
(LXGGXGDDX)
Hydrophobic helix
Amphipathic helix
Secretion
Nodulation
NodO wt
NodO E224-A260
NodO D188-T260
NodO G165-A260
NodO D127-A260
NodO H259GI
NodO I21-D167
224
260
188
165
127
259
GI
21
167
+
+++
-
(+)

Fig. 1. Model of NodO showing deleted derivatives and their properties

had the ability to secrete the protease PrtB, (this protein is normally secreted by the *prtDEF* gene products which are analogous to the haemolysin secretion system HlyBD-TolC). They concluded that NodO was probably secreted by a similar system and that the genes encoding such a secretion system were not located on the symbiotic plasmid since secretion of NodO or the protease was normal in Sym⁻ strains (Crank, Downie,1994; Scheu *et al.*, 1992)

In an attempt to identify the NodO secretion system, we have screened for Tn*5*-induced mutants of *R. leguminosarum* unable to secrete NodO. One such mutant was identified as being totally unable to secrete NodO. The Tn*5* insertion from this mutant was subcloned and the DNA sequence adjacent to the site of Tn*5* insertion was determined. This revealed strong similarity to the DNA sequence of genes that encode an ATP-binding cassette transporter, typical of the type of gene that we anticipated would be required for NodO secretion. It appears that mutations of this gene strongly influence nodulation of pea in a strain of *R.l. viciae* also carrying a mutation in *nodE.*

4. Biochemical role of NodO

NodO protein purified from the growth-medium supernatant of an over-producing strain was found to bind to liposomes and to form cation-selective channels in artificial planar lipid bilayers (Sutton *et al.*, 1994). This taken together with the observations that NodO is secreted into the growth medium and does not remain attached to *Rhizobium* (Scheu *et al.*, 1992) suggests that NodO may stimulate nodule formation by forming pores in plant membranes.

As shown in Fig. 1, a series of deleted derivatives of NodO has been constructed and these are secreted normally. These deleted forms of NodO were isolated from the growth-medium supernatants of appropriate strains and tested for their abilities to form pores in membranes. Only the derivative of NodO with the smallest internal deletion retained the ability to form pores, and those pores formed by that protein were very unstable. This suggested that deletion of residues 225-259 of NodO does not directly affect the pore-forming channel but may affect the way NodO interacts with artificial membranes. The larger internal deletions all remove a hydrophobic domain that is predicted to be α-helical. We conclude that this region is likely to be involved in pore formation. We have now constructed site-directed mutations that are expected to break this α-helical region.

5. NodO determines host specificity

Economou *et al.* (1995) showed that the *nodO* gene from *R.l. viciae* could confer on a *nodE* mutant of *R.l. trifoli*, ANU927 the ability to nodulate vetch. We have used this transfer of host specificity as an assay to monitor the biological activity of mutant forms of NodO in plant nodulation tests.

Significantly the deleted form of NodO that retained some pore-forming characteristics conferred on *R.l. trifoli* strain ANU927 a low but significant level of nodulation on vetch. In contrast strains of ANU927 carrying derivatives of *nodO* encoding proteins unable to form pores were unable to form nodules. Thus there is a high correlation between the ability of NodO to form pores and its ability to extend nodulation of vetch to *R.l. trifoli* strain ANU927.

Those derivatives of *nodO* carrying site-directed mutations that are predicted to break the α-helical regions of NodO were unable to confer vetch nodulations to ANU927. This indicates that these proposed α-helical regions do have an important role in the biological activity of NodO.

6. Discussion

The establishment of an effective symbiosis involves a complex series of interactions at the surface of the legume root. The principal signalling molecule produced by the bacteria can induce a depolarisation of root hair membranes (Ehrhardt *et al.*, 1992), which may prove to be a key stage in the infection process. The ability of a second signalling molecule, NodO from *R. l. viciae*, to cause ion movements in an artificial system may provide information concerning this stage of nodule initiation.

Although to date *nodO* has only been described in *R. l. viciae*, it seems likely that other rhizobial strains will produce signalling proteins that may have similar functions (Krishnan and Pueppke, 1993; van Rhijn and Vanderleyden, 1995). The ability of all *Rhizobium* strains tested to secrete NodO indicates the presence of a family of related proteins of unknown function. The identification of a mutant that is unable to secrete NodO (and probably other proteins) will elucidate the role of any related proteins of unknown function.

The nodulation phenotype of the NodO-secretion mutant in a *nodE* background of *R. l. viciae* demonstrates that the function of NodO is outside the bacteria. Given that no NodO reassociates with the *Rhizobium* cell following secretion (Scheu *et al.*, 1992), it suggests that the role of the protein involves an interaction with the plant cell.

The biochemical function of NodO has been analysed using a series of deletions and site-directed mutations. NodO is not a typical pore-forming protein, lacking in extensive hydrophobic domain as found in HlyA and other related toxins (Welch, 1990). However, the deletion of one of a pair of predicted α-helices severely reduces the ability of the protein to form ion channels, and concomitantly reduces its function in extending host-range. The removal of the second helix or the introduction of site-directed mutations that would disrupt either of the two helices completely abolishes NodO's ability to extend host-range.

The role of NodO in plant infection probably involves the formation of pores in a plant membrane. The site of the insertion, and the nature of molecule which passes through the pore (an ion or a Nod factor?) remains to be proven but will be of significance in relation to the broader issue of *nod*-factor signalling.

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THE GENETICS OF RHIZOBIUM HOST RANGE CONTROL: ALLELIC AND NON-ALLELIC VARIATION

F. Debellé *(1), P. Roche *(2), C. Plazanet (1), F. Maillet (1), C. Pujol (1), M. Ardourel (1), N. Demont (2), C. Rosenberg (1), G. Truchet (1), J.C. Promé (2), and J. Dénarié (1)
(1) Laboratoire de Biologie Moléculaire des Relations Plantes-Microorganismes CNRS-INRA, BP27, 31326 Castanet-Tolosan Cedex , France
(2) Laboratoire de Pharmacologie et de Toxicologie Fondamentales CNRS, 205 route de Narbonne, 31077 Toulouse Cedex
* The first two authors contributed equally to this work

The symbiotic association between Rhizobium and legumes is specific. For example *R. meliloti* nodulates *Medicago*, *Melilotus* and *Trigonella* species, *R. tropici* nodulates *Phaseolus* and *Leucaena*, and *R. leguminosarum* bv *viciae* 's hosts belong to the *Vicia* and *Pisum* genera (van Rhijn, Vanderleyden, 1995). The bacterial genes controlling nodulation and host specificity (*nod*, *nol* and *noe* genes) have been classified into 3 groups: (i) The regulatory *nodD* genes activate the expression of the other nodulation genes in response to compounds secreted by the host plants. (ii) The common *nodABC* genes are found in all rhizobial species studied so far and are considered as functionally conserved between these species. (iii) The species-specific *nod* genes, such as *nodH*, *nodL* or *nodFE* in *R. meliloti*, determine the host range. Mutations in the latter genes generally result in an alteration of the host spectrum. In addition, these genes are required for transferring the host range between different rhizobial species.
The common and host specific *nod* genes are involved in the synthesis of chito-lipooligosaccharide signals, the Nod factors, which act as host specific symbiotic signals during infection and nodule formation (Dénarié, Cullimore, 1993). The backbone of all the Nod factors consists of an N-acetylglucosamine oligosaccharide varying in length from 3 to 5 residues and substituted by an N-acyl chain at the non-reducing end. Its synthesis is directed by the common NodA, NodB and NodC proteins which are thought to function as an N-acyl transferase, a chito-oligosaccharide deacetylase and an N-acetylglucosaminyltransferase, respectively (Atkinson et al., 1994; Rohrig et al., 1994; John et al., 1993; Geremia et al., 1994, Spaink et al. 1994). In this paper we study the genetic and biochemical mechanisms which are responsible for the variations in Nod factor structure that make them host-specific. We show that not only the host specific, but also the common *nod* genes contribute to this variation.

Host range determination by non-allelic variation: the *nodH* and *nodL* genes

Non-allelic variation of species-specific *nod* genes is frequently observed among rhizobia. For example, the *nodH* gene is found in *R. meliloti*, but not in *R. l.* bv *viciae*. Mutations in *nodH* prevent *R. meliloti* from nodulating alfalfa, its normal host, but enable

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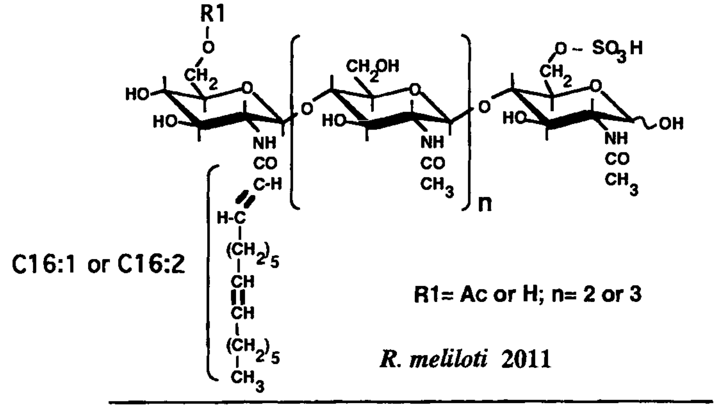
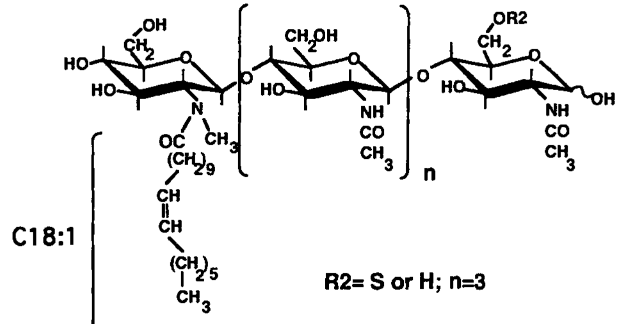
it to nodulate vetch, a host of *R. l.* bv *viciae* (Debellé et al., 1986; Horvath et al. 1986). *nodH* is required to transfer to both *R. l.* bv *viciae* and *R. l.* bv *trifolii* the ability to nodulate alfalfa (Faucher et al., 1989)). Thus *nodH* is an important host range determinant. Genetic and biochemical studies have shown that NodH is a sulfotransferase responsible for the sulfation of *R. meliloti* Nod factors (Roche et al., 1991; Atkinson et al., 1994; Bourdineaud et al., 1994; Schultze et al., 1995). Non-sulfated Nod factors produced by *nodH* mutants are active on vetch but not on alfalfa. This shows the importance of the sulfate substitution for Nod factor activity on alfalfa.



R. meliloti 2011



R. tropici CFN299

Fig.1: Structure of *R. meliloti* and *R. tropici* Nod factors

O-sulfation of the reducing glucosamine residue is not the only substitution important for alfalfa nodulation. For example, *R. tropici* produces sulfated Nod factors (Poupot et al., 1993), but does not nodulate alfalfa. Major differences between Nod factors from these two species are the following (Fig. 1): The *R. tropici* factors (i) are not O-acetylated, (ii) have vaccenic acid (C18:1) as N-acyl chain and not the C16 unsaturated fatty acids, and (iii) have a backbone of chitin pentamers. To transfer to *R. tropici* the ability to nodulate alfalfa we first introduced into *R. tropici* the pGMI149 plasmid which carries the regulatory *nodD1*, *nodD3* and *syrM* genes, and the structural *nodABC, nodFEG, nodH* and *nodPQ* genes. Only weak nodulation was observed. When a second plasmid, carrying the *R. meliloti nodL* gene, was also introduced, efficient nodulation was achieved. This is another example of host range determination by non-allelic variation: the *nodL* gene which is not present in *R. tropici* is required to transfer to *R. tropici* the ability to nodulate alfalfa. We and other laboratories (Ardourel et al., 1995; Bloemberg et al.,

1994) have shown that NodL is an acetyltransferase. When *nodL* is transferred to *R. tropici*, the transconjugant becomes able to produce O-acetylated Nod factors more active on alfalfa than the non-acetylated ones.

Host range determination by allelic variation

Some of the host range genes are present as allelic variants in different rhizobial strains. For example, the *nodFE* genes are found in *R. meliloti* and *R. l. bv viciae,* and play an important role in the control of host specificity in both species. In spite of their highly conserved nucleotide sequences, these genes are not functionally conserved between the 2 species: mutations in *R. meliloti nodFE* cannot be complemented by the *R. l.* bv *viciae nodFE* genes. The *nodFE* genes were first shown, in *R. l.* bv. *viciae*, to specify the synthesis of a highly unsaturated C18:4 fatty acid (Spaink et al., 1991). In contrast, in *R. meliloti*, Nod factors are N-acylated by C16 unsaturated fatty acids (C16:1, C16:2 and C16:3) (Lerouge et al., 1990; Schultze et al., 1992; Demont et al., 1993). In *R. meliloti*, downstream to *nodFE* there is an additional gene, *nodG*, which encodes a protein homologous to ketoacyl reductases (Sheldon et al., 1990). We have shown that *nodG* is not required for the synthesis of the Nod factor acyl group and that the *R. meliloti* and *R. l.* bv *viciae* NodFE proteins specify the synthesis of acyl chains which differ in length and degree of unsaturation (Demont et al., 1993). These acyl chains contribute to the specificity of the Nod factors they substitute.

The *nodABC* genes are present in all rhizobial strains. On the basis of functional complementation tests they were considered as interchangeable between rhizobial strains. However, our recent genetic analysis of the mechanisms responsible for the differences in Nod factor structure and host specificity between *R. tropici* and *R. meliloti*, led us to reinvestigate this question. We first analysed the Nod factors produced by the *R. tropici* (pGMI149, pnodL) transconjugant that is able to nodulate alfalfa. Separation of Nod factors on a reverse phase HPLC column showed a multiplicity of partly resolved peaks. Acid hydrolysis was performed on the mixture of Nod factors and the liberated fatty acids were analysed by GC, by comparison with known standards. Contrary to wild-type *R. tropici*, the *R. tropici* (pGMI149, pnodL) hybrid strain produced Nod factors (i) substituted by C16 unsaturated fatty acids (C16:1, C16:2) in addition to those substituted by the regular C18:1 fatty acid, and (ii) with a certain proportion of glucosamine tetramers, in addition to pentamers. In order to identify the genes required for the synthesis of Nod factors with 4 glucosamine residues or substituted by C16 unsaturated fatty acids we introduced Tn5 insertions in the *nod* genes cloned on the pGMI149. A Tn5 insertion in *nodA* prevented the production of Nod factors substituted by C16 unsaturated fatty acids, and decreased the proportion of tetramers. This indicates that the *R. meliloti nodABC* operon is required for the synthesis of Nod factors substituted by C16 unsaturated acyl chains and has an influence on the length of the chitin oligomer backbone. The multiplicity of Nod factors produced by the *R. tropici* (pGMI149, pnodL) derivatives made it difficult to purify the different molecules to study them in more detail. Therefore, in order to understand better why the *R. tropici* and *R. meliloti nodABC* genes are functionally different, we decided to individually exchange these genes between the two species.

Strategies for studying the respective roles of *nodA*, *nodB* and *nodC*

To introduce the *R. tropici nodABC* genes into *R. meliloti* we first had to clone these genes. A partial library of *R. tropici* CFN299 DNA was screened with a *R. meliloti nodC* probe. A 6 kb *Eco*RI restriction fragment carrying *nodABCSU* was isolated (Fig. 2). The nucleotide sequence of the *nodABC* genes was determined. The organization of the *nodD1ABCSU* genes in the *R. tropici* CFN299 (type IIA) and *R. tropici* CIAT899 (type IIB) strains is similar and their nucleotide sequence is highly conserved (Waelkens et al., 1995). To make sure that the *R. tropici nod* genes would be expressed in an *R. meliloti* heterologous background, the *nodA* , *nodC* and *nodABC* genes were cloned under the control of the constitutive pNm promoter in an IncQ group cloning vector (Labes et al., 1990). As controls, the *R. meliloti nodA, nodC* ,and *nodABC* genes were cloned into the same expression vector. An IncQ plasmid was chosen for this to allow the further introduction of the regulatory nodD3/syrM genes on an IncP plasmid.

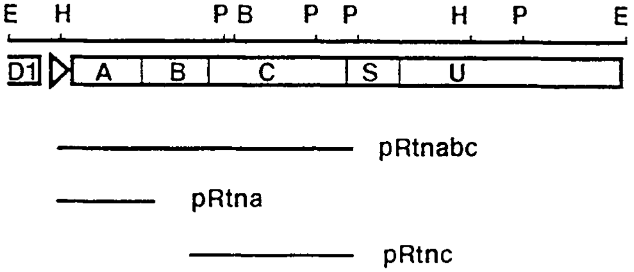


Fig. 2: Physical and genetic map of *R. tropici* nodulation genes

In addition, a non-polar *nodA* mutation was constructed in *R. meliloti* as follows: A DNA fragment carrying the *nodAB* region was cloned into the M13mp19 bacteriophage. A deletion was introduced into the *nodA* gene, conserving the reading frame. The deleted *nodAB* region was then recloned into the suicide plasmid pJQ200 (Quandt et al., 1993), carrying the *sacB* gene. After introduction of this plasmid into *R. meliloti*, a first recombination event resulted in the integration of the suicide plasmid into the *R. meliloti* pSym. By growing the transconjugant on medium with saccharose, we selected for a second recombination event allowing the replacement of the wild-type *nodA* by the deleted gene.

The *R. meliloti* and *R. tropici nodA* genes specify the Nod factor N-acylation by different fatty acids

First, the *R. tropici nodABC* genes were introduced into *R. meliloti* strains having Tn5 insertions in the *nodA*, *nodB*, or *nodC* genes. To increase Nod factor production, the plasmid pMH682 carrying the regulatory *nodD3* and *syrM* genes was also introduced into the mutant strains. The Nod factors produced by the hybrid strains were then extracted, purified and analysed. When the *R. tropici nodABC* genes were introduced into an *R. meliloti nodA::Tn5* mutant, ie when the *R.meliloti nodABC* genes were replaced by the *R. tropici nodABC*, the Nod factors were acylated by vaccenic acid (C18:1). In contrast, when the *R. tropici nodABC* genes were introduced in a *R. meliloti nodB* mutant (having an intact *nodA* gene) some N-acylation by unsaturated C16 fatty acids could be observed. These experiments suggested that the *R. meliloti nodA* gene is required for the synthesis of Nod factors substituted by C16 unsaturated fatty acids; *nodA* of *R. meliloti* cannot be replaced by the *R. tropici nodA* gene in this function.

In order to further analyse the role of *nodA* in the control of the nature of the acyl substituents, an *R. meliloti* derivative was constructed in which the *nodA* gene was replaced by its *R. tropici* counterpart. A *R. meliloti* derivative carrying a non-polar *nodA* mutation was used as a recipient, to ensure a normal expression of the downstream *nodBC* and *nodIJ* genes. These experiments clearly showed that the *R. tropici nodA* gene specifies the synthesis of Nod factors acylated with vaccenic acid and cannot replace its *R. meliloti* counterpart to substitute the Nod factor chito-oligosaccharide backbone by C16 unsaturated fatty acids. The NodA protein is thought to be an N-acyltransferase. Contrary to *R. tropici* NodA, the *R. meliloti* NodA might recognize C16 unsaturated fatty acids. Alternatively, the NodA protein could recognize a specific acyl carrier protein, such as that encoded by the *R. meliloti nodF* gene.

The *R. meliloti and R. tropici nodC* genes specify the synthesis of chitin oligomers of different chain length

When the *R. tropici nodABC* genes were introduced into an *R. meliloti nodC* mutant, the major Nod factors produced had 5 glucosamine residues instead of 4, as in the case of *R. meliloti*. Thus the *R. meliloti nodC* gene might be required for the production of Nod factors with 4 glucosamine residues. The importance of *nodA* and *nodB* in the control of the length of the chitin oligomer backbone could not be established since the mutations by Tn5 insertion in *nodA* and *nodB*, which prevent the synthesis of Nod factors with 4 glucosamine residues, could have a polar effect on the downstream *nodC* gene.

In order to analyse the role of *nodC* in the control of the oligosaccharide chain length, an *R. meliloti* derivative was constructed in which the *nodC* gene was replaced by its *R. tropici* counterpart. A plasmid carrying the *R. tropici nodC* gene was introduced into a *nodC*::Tn5 *R. meliloti* mutant. Replacing the *R. meliloti nodC* gene by its *R. tropici* counterpart resulted in the production of Nod factors mostly with 5 glucosamine residues (the chain length characteristic of *R. tropici* Nod factors) instead of Nod factors mostly with 4 glucosamine residues that are produced by wild-type *R. meliloti*. This shows that *nodC* is the main determinant of the difference in chito-oligosaccharide chain length between *R. tropici* and *R. meliloti*. Thus NodC is an N-acetylglucosaminyl transferase that is also able to control the length of the final chitin oligomer.

Conclusion

In this work we have shown that, contrary to what was generally thought, the common *nodABC* genes are not functionally conserved between all rhizobial species. Different alleles of *nodC* can specify the synthesis of Nod factors having different chito-oligosaccharide chain length. In addition, the different *nodA* alleles control the substitution of Nod factors by different acyl chains. Thus, just like the host specific *nodFE* genes, the common *nodABC* genes contribute to the determination of Nod factor diversity by a mechanism of allelic variation. In future work we will study the biological activity of the Nod factors produced by *R. meliloti* strains in which the *nodA* or *nodC* genes have been replaced by their *R. tropici* counterparts. Previous work has already shown that Nod factors substituted by C18:1 fatty acids produced by *R. meliloti nodFE* mutants are less active on alfalfa than Nod factors produced by the wild-type strain (Ardourel et al., 1994). In addition, the Nod factors having 5 glucosamine residues produced by *R. meliloti* are 100 times less active than those having 4 glucosamine residues (Schultze et al., 1992). This indicates that the *nodA* and *nodC* genes, by controlling the Nod factor oligosaccharide chain length and the nature of the acyl sustituent might be important determinants of the rhizobial host range. This is consistent with the recent observation that the phylogenetic trees constructed to represent NodC homologies between different rhizobial species do not correspond to the phylogenetic trees prepared with 16S ribosomal RNA sequences, that is to the taxonomic positions of the bacterial strains. Instead, NodC evolutionary trees are similar to the trees describing molecular evolution of the leguminous hosts. All these data indicate that the common *nodABC* genes have evolved, as the species-specific *nod* genes, for adaptation to particular leguminous hosts.

Acknowledgments

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NOD METABOLITES AND OTHER COMPOUNDS EXCRETED BY *RHIZOBIUM* SPP.

MARTINEZ, E.[1], LAEREMANS, T.[1], POUPOT, R.[2], ROGEL, M.A.[1], LOPEZ, L.[1], GARCIA, F.[3], VANDERLEYDEN, J.[4], PROME, J.C.[2], LARA, F.[3]. [1]CIFN-UNAM. Ap.P. 565-A, Cuernavaca, Mor., México; [2]Lab. Pharmacologie et Toxicologie Fondamentales CNRS, 31077 Toulouse Cedex, France; [3]Instituto de Química, UNAM. México, D.F.; [4]K. Universiteit, Leuven, Belgium.

1. INTRODUCTION

The formation of a nitrogen fixing nodule is the result of a successful communication process between plants and bacteria. In response to root exudates or flavonoids, the *Rhizobium* species produce lipo-oligosaccharides, or Nod factors, that are responsible of eliciting meristems and nodule-like structures in roots. From the discovery of Nod factors in *R. meliloti* (Lerouge et al., 1990), different research groups have defined the corresponding chemical structures from Nod metabolites produced by different *Rhizobium* strains (Bec-Ferté et al., 1993; Carlson et al., 1993; Firmin et al., 1993; López-Lara et al., 1995; Mergaert et al., 1993; Price et al., 1992; Sanjuan et al., 1992; Schultze et al., 1992; Spaink et al., 1991).

Based on the analysis of *Phaseolus vulgaris* bean *Rhizobium* populations, we established that different genetic *Rhizobium* lineages were capable of nodulating bean (Hernández-Lucas et al., 1995; Piñero et al., 1988), and some of them have even been recognized as different species, *R. etli* (Segovia et al., 1993) and *R. tropici* (Martínez-Romero et al., 1991). In an attempt to explore if bean specificity could be related to a common Nod factor produced by bean rhizobia, we addressed part of our research efforts to define the structure of Nod metabolites from wild type *R. etli* and from wild type *R. tropici.*

2. RESULTS AND DISCUSSION

Both *R. tropici* and *R. etli* are broad host range bacteria that share the following legume hosts, *Gliricidia maculata*, *Clianthus formosus*, *Crotaria sericia* (Hernández-Lucas et al., 1995), in addition to *Phaseolus vulgaris* bean. *R. tropici* has been subdivided in two types or subtypes (Martínez-Romero et al., 1991) that have a number of important differences including recently described type-specific megaplasmids (Geniaux et al., 1995).

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Two of the major compounds produced by wild type *R. tropici* type A strain CFN299 are pentamers of *N*-acetyl glucosamine substituted with an *N*-methyl-*N*-vaccenic group (Poupot et al., 1993) either with or without a sulfate at the 6-terminal reducing end of the oligosaccharide. The molecule resembles *R. meliloti* Nod factors, which have a sulfate at the same position. In *R. meliloti*, the sulfate modification is related to host specificity since mutants in *nodH* and double mutants in *nodP* and *nodQ* are Nod$^-$ or greatly impaired in their nodulation capacity in alfalfa (Debellé et al., 1986; Horvath et al., 1986; Ogawa et al., 1991; Swanson et al., 1987; Schwedock, Long, 1992).

To analyze the role of the sulfate modification in bean nodulation, we evaluated *in vitro* the effects of both purified compounds (at concentrations of 10^{-5}, 10^{-6} and 10^{-7} M) on axenic plants grown in agar flasks. Nodule-like structures were formed on a majority of plants treated with the sulfated, but not with the non-sulfated molecules, although the latter were able to elicit meristem activity in bean roots (Fig. 1).

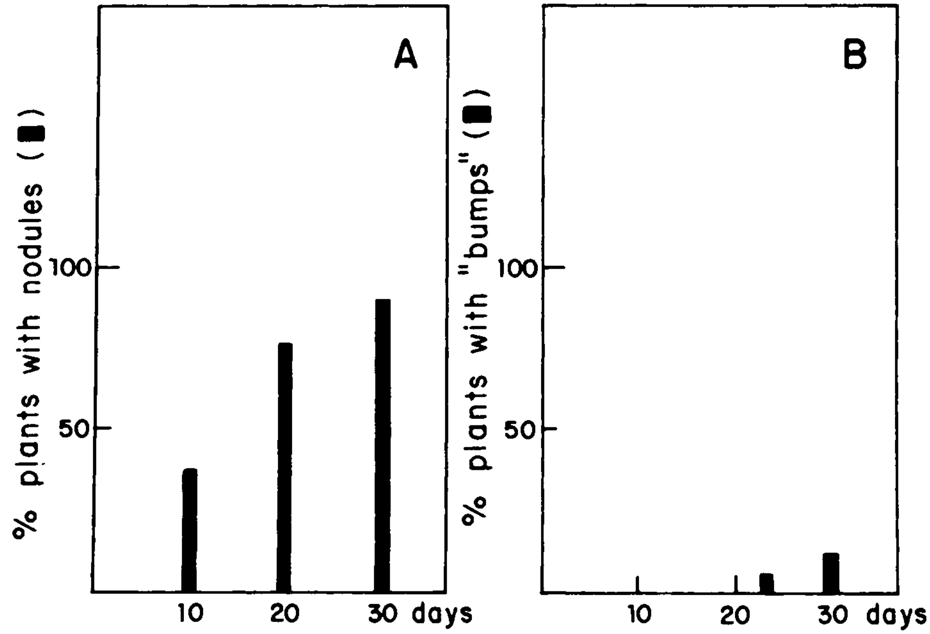


Fig. 1 Percentage of *P. vulgaris* bean plants that developed nodule-like structures induced by Nod metabolites at 10^{-6} M as a function of time. A) Assays with the sulfated factor Nod Rt-V [Me, S]. B) Assays with the non-sulfated factor Nod Rt-V [Me]. Thirty plants per assay in four independent experiments were considered.

To evaluate the role of the sulfate group in the nodulation process induced by the bacteria, we analyzed the genes corresponding to those responsible for sulfation in *R. meliloti* (Roche et al., 1991; Schwedock, Long, 1990; 1992). We have cloned and sequenced *nodH*, *nodP* and *nodQ* from *R. tropici* strain CFN299. In CFN299 only a single copy of *nodPQ* is found. An insertion mutant in *nodP* has been obtained (CFNE200) which carries a Km cassette. CFNE200 does not produce sulfated molecules *in vitro* when analyzed by TLC chromatography using ^{35}S-sulfate. Nodulation assays were performed as described (Martínez-Romero, Rosenblueth, 1990) in the standard Negro Xamapa bean cultivar and in cultivar N18 116, which has been selected for its high nitrogen fixing capacity. In Negro Xamapa, CFNE200 had a diminished nodulation capacity, producing around 60-70% of the nodules compared to the wild type in agar flasks. Similarly, in N-8-116 bean plants grown in flasks with vermiculite, CFNE200 formed 65% of the nodules compared to the wild type, and the nodules were smaller. For *P. vulgaris* bean nodulation, the sulfate in *R. tropici* Nod metabolites seems to be import both *in vitro* and *in vivo*.

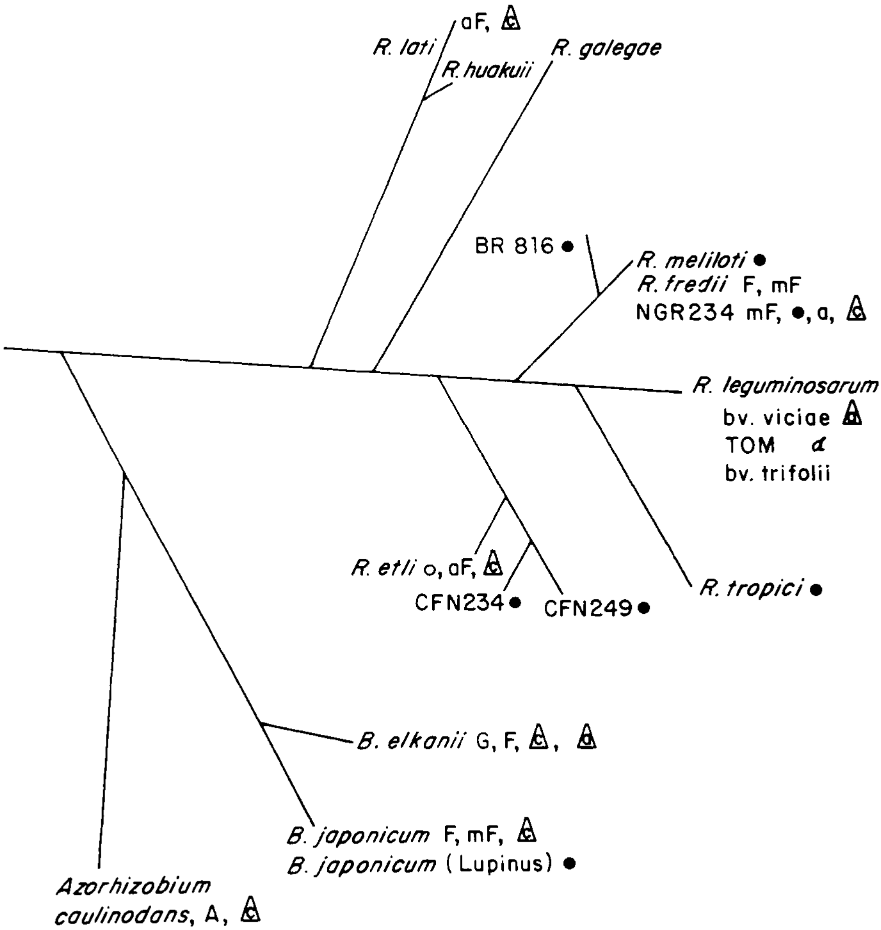
R. etli (etl=bean in náhuatl, the aztec language) is the bacteria that is normally encountered in bean nodules in agricultural fields in Mesoamerica (Segovia et al., 1993). *R. etli* Nod factors (Poupot et al., 1995) have both common and different structural features when compared to *R. tropici* Nod factors. Both are pentamers and they contain *N*-methylated cis-vaccenic acid; but they differ by having a 4-O-acetyl-fucose instead of sulfate at the reducing end, and both carbamoylated and non carbamoylated molecules have been found in *R. etli*. A mixture of both types of molecules is capable of inducing the formation of nodule-like structures in bean plants (unpublished). The presence of sulfate in *R. etli* Nod factors was carefully searched and we found no sulfated group in *R. etli* Nod metabolites (Poupot et al., 1995).

We conclude from these results that Nod factors with different chemical structures are active on bean and that pentameric molecules with N-methyl substituents may be determinants for bean nodulation, nevertheless this is a very common type of structure also found in Nod factors from other specificities (Carlson et al., 1993; López-Lara et al., 1995; Mergaert et al., 1993; Price et al., 1992).

The structure of *R. loti* Nod factor was recently reported (López-Lara et al., 1995). The lipo-oligosaccharides produced by *R. etli* and by *R. loti* are amazingly similar. From both species the molecules are *N*-acetylglucosamine pentasaccharides *N*-methyl-*N*-vaccenoylated at their non-reducing end and with 4-*O* acetyl fucose at the reducing terminal residue. A carbamoyl group has been described on the terminal non-reducing saccharide residue. The exact position of the carbamoyl group has not been defined in *R. etli* and it may be that its position is important for specificity. In view of these similarities, we tested *R. etli* strain CFN42 for *Lotus corniculatus* nodulation and *R. loti* strain NZP2037 for bean nodulation. We found that *R. etli* strain CFN42 was capable of forming ineffective nodules in *Lotus corniculatus* (Hernández-Lucas et al., 1995), and that *R. loti* strain NZP2037 formed very scarce and ineffective nodules in bean. This suggests that the cross nodulation is due to the similarities of the Nod factors. It would be interesting to explore what *R. etli* requires to be able to form nitrogen fixing nodules in *L. corniculatus*.

From our results and from others reported in the literature, it is becoming evident that Nod factors substituents and the genes responsible are not so species-specific as was previously thought as they may be shared by bacteria with different host ranges. In an attempt to explore the evolutionary trends of Nod factor decoration we have analyzed the chemical modifications of the Nod factors in the frame of the phylogenetic relationships of *Rhizobium* species. In a dendrogram based on the analysis of 16S rRNA gene sequences (Martínez-Romero, 1994) we have identified the chemical modifications that have been reported from Nod factors (Fig. 2). There is no correlation between the *Rhizobium* chromosomal lineages and the type of modifications on the lipo-oligosaccharides that the bacteria produce. For example, sulfate, fucose and its acetylated or methylated forms at the reducing saccharidic end are scattered among different branches with no clear relationship to specificity. In bacteria, in other cases, genes that are scattered among chromosomal lineages are subject to strong selective pressures, e.g. antigenic determinants in Enterobacteria (Smith et al., 1990). The role of the sulfate modification to protect or to stimulate *R. meliloti* Nod factor from chitinase degradation has been demonstrated (Staehelin et al., 1994). Perhaps the Nod factor substituents have to change as fast as chitinase specificities change in response to plant pathogens.

In the scheme presented, the lipid moiety has not been considered since there are indications that it may play a role in the ability of the Nod factor to enter the plant cell (Spaink et al., 1994) perhaps by aiding dissolution in the cell membranes. If this is the case, an interesting correlation arises: temperature is a major environmental factor governing the distribution of both wild and cultivated plant species. The plant fatty acid composition in the Leguminosae was correlated with the latitude of plant origin. Saturation is more prevalent in tropical legumes with higher chilling sensitivities (Matsuo et al., 1992). It could be that *Rhizobium* Nod factors have to match the corresponding fatty acid composition of their host plant membranes. Furthermore, in *E. coli* and *Salmonella* fatty acid saturation is clearly related to growth temperature (van Alphen et al., 1979; Wollenweber et al., 1983), and unsaturated lipids have been found in the lipo-oligosaccharides from *R. meliloti* and from *R. leguminosarum* bv. viciae whose hosts are from temperate regions.


oF, Δ
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R. huakuii
R. galegae
BR 816 ●
R. meliloti ●
R. fredii F, mF
NGR234 mF, ●, a, Δ
R. leguminosarum
bv. viciae Δ
TOM α
bv. trifolii
R. etli o, aF, Δ
CFN234 ●
CFN249 ●
R. tropici ●
B. elkanii G, F, Δ, Δ
B. japonicum F, mF, Δ
B. japonicum (Lupinus) ●
Azorhizobium
caulinodans, A, Δ

Fig. 2. Dendrogram indicating *Rhizobium* phylogenetic relationships inferred from 16S ribosomal RNA sequences. The *Rhizobium* species in the figure are those which include strains found to produce ● sulfated; F, fucose-substituted; aF, acetylfucose-substituted; mF, methyl-fucose-substituted; a, α acetylated; A, arabinose-modified; c, carbamoyl-substituted; G glycerol-linked Nod metabolites. Δ indicates that the substitution is at the non-reducing saccharidic end, while all others are at the reducing-end. The presence of sulfate in *Rhizobium* spp strains CFN234, CFN249, and BR816 and in *B. japonicus* (*Lupinus*) has been determined by the incorporation of S^{35} in lipo-oligosaccharides analyzed by TLC chromatography in CIFN (unpublished).

We have found that in addition to Nod factors, *Rhizobium* species produce and excrete several structurally related aromatic molecules when the bacteria are grown in minimal medium. We are presently analyzing the chemical structure of these compounds. All the *Rhizobium* strains tested from different species produce them and some of these compounds are species specific.

A species - specific compound produced by *R. tropici* type A strain CFN299 supplemented with tryptophan is a novel type of conjugated auxin not encoded on the symbiotic plasmid. Bean roots readily absorb it and it causes extensive root-curling, branching and thick short roots. We propose that some of these newly described compounds may constitute additional signal molecules in plant-bacteria communication.

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METABOLISM OF FLAVONOIDS BY RHIZOBIA

COOPER, J.E.[1], RAO, J.R.[1], EVERAERT, E.[2], DE COOMAN, L.[2]
[1]Department of Applied Plant Science, The Queen's University of Belfast, Newforge Lane, Belfast, BT9 5PX, Northern Ireland, UK.
[2]Laboratory of Plant Biochemistry, Rijksuniversiteit Gent, KL Ledeganckstraat 35, B-9000, Gent, Belgium.

Introduction

In common with many other soil microorganisms rhizobia are capable of catabolizing a variety of aromatic compounds. Monocylic aromatics and hydroaromatics such as benzoate and shikimate can be degraded to 3-oxoadipate via catechol or protocatechuate prior to entry into the tricarboxylic acid cycle (Chen et al., 1984; Parke, Ornston, 1984) and protocatechuate itself is a universal growth substrate for rhizobia. The possibility that rhizobia could catabolize polycyclic flavonoids responsible for *nod* gene induction has been raised as a consequence of experiments which showed that such compounds also induced expression of *Rhizobium* genes with unknown functions and showing no homologies with nodulation gene promoters (Sadowsky et al., 1988; Perret et al., 1994). The first demonstration of flavonoid catabolism by *Rhizobium* involved utilization of the flavan-3-ol catechin by an isolate from *Leucaena leucocephala* with attendant formation of phloroglucinol carboxylic acid and protocatechuate (Gajendiran, Mahadevan, 1988). Rao et al. (1991) subsequently reported that *R. loti* could cleave the C-ring of the pentahydroxy flavone quercetin by means of a novel mechanism which yielded phloroglucinol and protocatechuate among the degradation products. Further studies with other *Rhizobium* species/biovars and their respective *nod* gene inducers confirmed that flavonoid degradation is a common metabolic feature in members of this genus (Rao, Cooper, 1994). In this paper we report on recent developments from our studies of flavonoid metabolism by free-living rhizobia:

1) evidence for C-ring modification and fission during interactions between rhizobia and their flavonoid or isoflavonoid *nod* gene inducers;

2) the establishment of a contribution from carbon atoms of *nod* gene-inducing naringenin to the structure of *R. leguminosarum* bv viciae Nod factor Rlv-IV.

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Flavonoid degradation by rhizobia- mechanisms and products

Flavonoids were dispensed in large volumes (≥ 1 litre) of mannitol or xylose-supplemented culture medium to give an initial concentration of 10μM. Rhizobia were added as single strain cultures at a starting density of 1.5×10^3 cells/ml. After incubation for periods up to 48h cultures were centrifuged and supernatants extracted prior to analysis GC-MS or HPLC. Details of methodology can be found in previous publications (Rao, Cooper, 1993, 1994). When incubated with rhizobia, flavonoids with OH substitutions at the 5 and 7 positions yielded phloroglucinol as the conserved A-ring product and those with a single OH substitution at the 7 position yielded resorcinol. A greater variety of conserved B-ring metabolites was detected including p-coumaric, p-hydroxybenzoic, protocatechuic, phenylacetic and caffeic acids. In all cases, degradation of the three ring structure was initiated by fissions in the central (C) ring. In the case of the flavanone naringenin, incubation for 2h with *R. leguminosarum* bv viciae permitted the transient detection by GC-MS of C-ring modification products, in the form of new chalcone (M^+314) and flavanone (M^+300) structures, in derivatized supernatant extracts. When incubated with *R. meliloti,* luteolin yielded several new closed C-ring metabolites despite the concurrent existence of C-ring fission and the formation of five monocyclic aromatic degradation products (Fig 1a).

The same basic pattern of degradation was also displayed when the isoflavone *nod* gene inducers daidzein and genistein were incubated with *Bradyrhizobium japonicum, R. fredii* or *Rhizobium* sp NGR234. For example, several C-ring modification products were detected when daidzein was incubated with *B. japonicum*; these included isoliquiritigenin (open C-ring) and liquiritigenin (closed C-ring). The principal C-ring fission products in this system were resorcinol (conserved A-ring product), 4-hydroxybenzoic acid and 4-hydroxycinnamic acid (conserved B-ring products). A presumed A-ring derivative, umbelliferone (or its satellite compound 4-methyl umbelliferone), was also detected in culture supernatant extracts. A proposed degradation pathway for daidzen is presented in Fig 1b. None of the principal A- or B-ring derived degradation products was detected in cell extracts but daidzein-treated *B. japonicum* cells did contain liquiritigenin, coumestrol and umbelliferone as well as unmetabolized daidzein. An HPLC chromatogram of bulked supernatant extracts from genistein-supplemented medium after incubation for 24h with *B. japonicum* is shown in Fig 2. HPLC protocols were based on those described by Graham (1991) and peak identities were obtained from comparisons of their UV absorption spectra and mass spectra from GC-MS analyses with those of authentic compounds.

It can now be concluded that a wide range of flavonoid and isoflavonoid *nod* gene inducers can be catabolized by rhizobia via similar C-ring fisson mechanisms. Products encompass both open and closed C-ring modification structures in addition to monocyclic hydroaromatics. The process can be viewed as a reversal of plant flavonoid biosynthesis. Flavonoid degradation via C-ring fission has been previously reported for only two other bacterial genera: *Eubacterium* and *Clostridium*

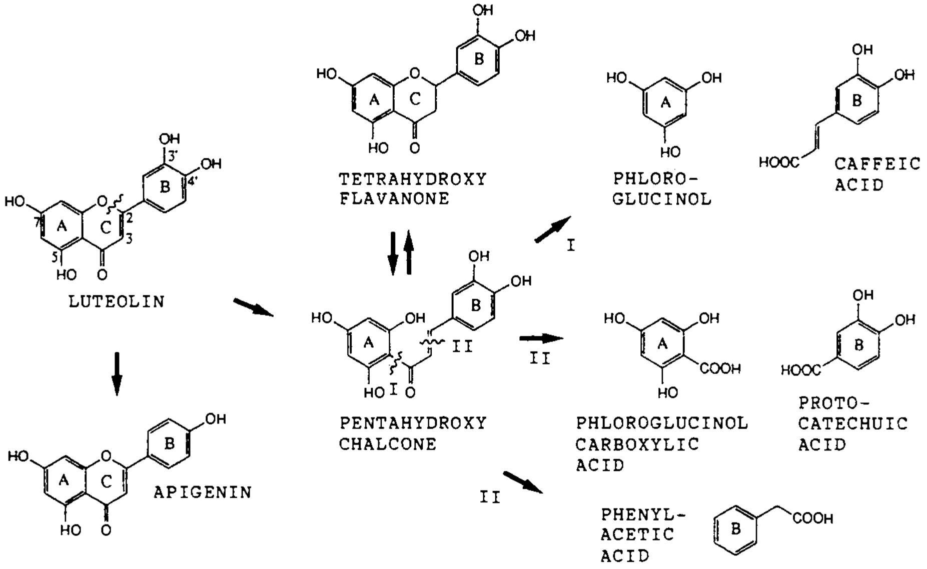
LUTEOLIN
APIGENIN
TETRAHYDROXY FLAVANONE
PENTAHYDROXY CHALCONE
PHLOROGLUCINOL
CAFFEIC ACID
PHLOROGLUCINOL CARBOXYLIC ACID
PROTOCATECHUIC ACID
PHENYLACETIC ACID
I
II
A
B
C
HO
OH
COOH
HOOC

Fig. 1a. Proposed degradation pathway for luteolin by *R meliloti*.

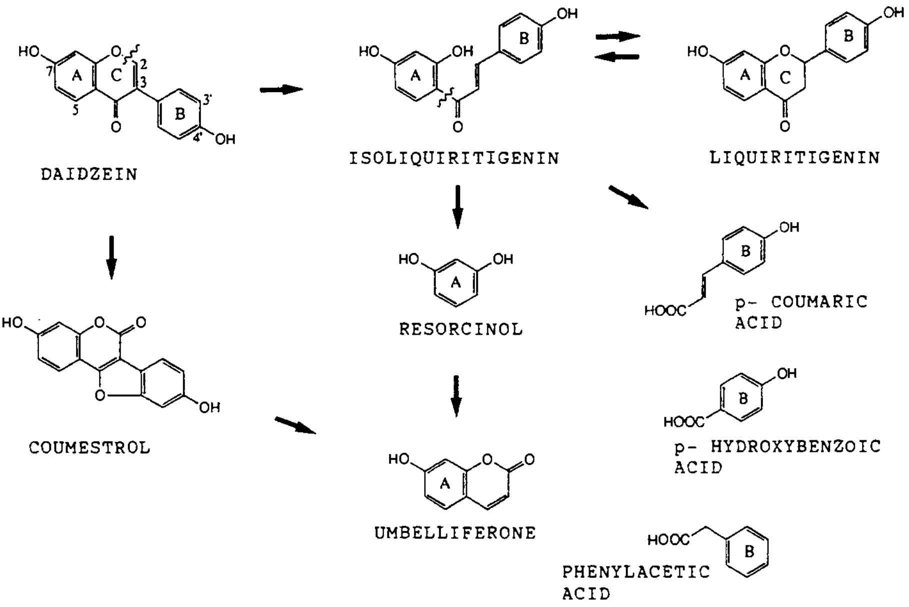
DAIDZEIN
COUMESTROL
ISOLIQUIRITIGENIN
LIQUIRITIGENIN
RESORCINOL
UMBELLIFERONE
p- COUMARIC ACID
p- HYDROXYBENZOIC ACID
PHENYLACETIC ACID
A
B
C
HO
OH
HOOC

Fig. 1b. Proposed degradation pathway for daidzein by *B. japonicum*.

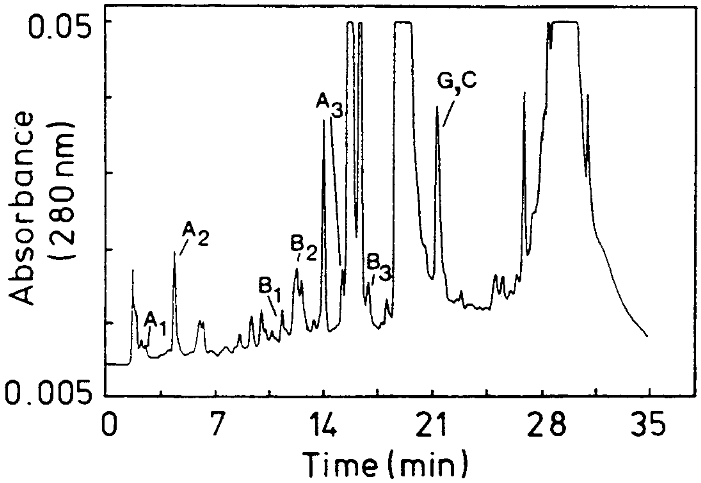

Fig. 2. HPLC chromatogram of genistein-supplemented medium after incubation with *B. japonicum* for 24 h. A1 = phloroglucinol; A2 = phloroglucinol carboxylic acid; A3 = umbelliferone; B1 = 4-hydroxybenzoic acid; B2 = p-coumaric acid; B3 = phenylacetic acid; G, C = residual genistein and coumestrol.

(Krumholz, Bryant, 1986; Winter et al., 1989). However, the ensuing degradation products were different from the rhizobial metabolites.

Our results raise several questions concerning the origin of previously reported new flavonoid structures in legume root exudates and the consequences of degradation for *nod* gene regulation. There is a remarkable similarity between the C-ring modification products from naringenin catabolism and the new chalcone and flavanone structures reported by Recourt et al. (1991) in *Vicia sativa* root exudates after inoculation with *R. leguminosarum*, suggesting that bacterial degradation, rather than *de novo* plant synthesis and release, could account for the occurrence of at least some of these compounds. The formation of inducer-derived metabolites could have implications for the control of *nod* gene induction in rhizobia. For example, isoliquiritigenin is a powerful inducer for *B. japonicum* (Kape et al., 1992) but umbelliferone is a potent *nod* gene inhibitor (Kosslak et al., 1990).

Metabolic fate of naringenin during Nod factor synthesis by *R. leguminosarum* bv viciae.

To trace the fate of an inducing flavonoid during Nod factor synthesis by *Rhizobium* ^{14}C-labelled naringenin was presented to *R. leguminosarum* bv viciae containing a wild type Sym plasmid (RBL 5560) and also to a Sym plasmid-cured derivative strain (LPR 5045). ^{14}C-labelled naringenin was synthesised enzymatically using the methods of De Cooman et al. (1993) to provide labelling in the A-ring of the compound. The highly purified labelled inducer [sp activity 6,259 kBq/µmol) was supplied at a concentration of 2 nM (ca 12.5 kBq) in the presence of cold acetate (0.5 µM). ^{14}C-acetate (sp. activity 2072 kBq/µmol) at a concentration of 0.5 µM (ca 1,036 kBq) together with cold naringenin (2 nM) were also supplied to the same two strains as a second, separate treatment. Culture conditions and TLC/HPLC separation of Nod metabolites were as described by Spaink et al. (1991). Extracts of a culture of RBL 5560 induced with cold naringenin (2 nM) in the presence of ^{14}C-acetate yielded the full range of Nod metabolites previously reported for this organism (Spaink et al., 1991, 1992). However, when supplied with ^{14}C-naringenin and cold acetate RBL 5560 gave a single clear signal on the autoradiogram of the TLC at the position corresponding to the two co-migrating Nod metabolites Rlv-IV (Ac, C18:4) and Rlv-V (Ac, C18:1) in the ^{14}C-acetate treatment. HPLC fractionation of Nod factors from the ^{14}C-naringenin treatment identified a strongly labelled metabolite with a UV absorbance maximum of 303 nm and a relative molecular mass (Mr) of 1088, consistent with the presence of Nod Rlv-IV (Ac, C18:4). After acid and alkali hydrolysis 95% of the label residing in the Nod factor was recovered from the lipid fraction. These findings confirm that naringenin is assimilated by *Rhizobium* when it is presented at *nod* gene inducing concentrations. They also show that carbon atoms from the inducer flavonoid supply a hitherto unsuspected component of Nod factor structure which is located in the highly unsaturated lipid moiety attached to the oligosaccharide backbone.

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PLANT SIGNALS TO SOIL MICROBES: REGULATORS OF RHIZOSPHERE COLONIZATION

PHILLIPS, D.A.(1), STREIT, W.R.(1), JOSEPH, C.M.(1), SANDE, E.(1), PALUMBO, J.D.(1,2), KADO, C.I.(2), and de BRUIJN, F.J.(3)
(1)Dept.of Agronomy and (2)Dept.of Plant Pathology, University of California, Davis, CA 95616; (3)Plant Research Laboratory and Dept. of Microbiology, Michigan State University, East Lansing, MI 48824

It is now well-established that natural plant products induce gene transcription in plant-associated soil bacteria. For example, nodulation (*nod, nol*) genes in *Rhizobium* and *Bradyrhizobium* as well as virulence (*vir*) genes in *Agrobacterium* are controlled by plant compounds (Long, Staskawicz, 1993). The list of plant flavonoids that regulate *nod* genes in rhizobia has now been extended to include diverse groups of flavonoids and flavonoid glycosides (Hungria et al., 1991; Smit et al, 1992; Phillips et al., 1994). In the case of *Rhizobium meliloti*, two *nod*-gene-inducing betaines, stachydrine and trigonelline, also are released from many *Medicago* species (Phillips et al., 1995). The availability of *nod*-gene-inducers and other compounds in soil around alfalfa roots (Léon-Barrios et al., 1993) suggests that applied ecological studies should now consider whether molecular signals from plants can affect other important microbial functions, such as growth and root colonization in soil. Understanding signal-response events that actually occur in soil should make it easier to introduce genetically improved rhizobia to soil containing indigenous strains and may enhance efforts to use other beneficial microbes that control plant disease, degrade pesticides, or deter invertebrate pests.

To identify key plant signals and bacterial genes that control root colonization, rigorous new approaches are needed. Many plant metabolites besides flavonoids may regulate soil bacteria. Those compounds include B-group vitamins (Rovira, Harris, 1961), betaines (Gloux, Le Rudulier, 1989; Boivin et al., 1991; Goldmann et al., 1994), rhizopines (Murphy et al., 1993; Saint et al., 1993), and calystegins (Tepfer et al., 1988). Unfortunately, however, ecologically relevant data are not available in all cases for these compounds. Two important points that should be established by such data include 1) the presence and amount of the compound in a plant-soil-microbe continuum and 2) quantification of the extent to which the compound affects root colonization. A direct method for achieving these objectives uses mutagenesis to produce bacteria that no longer respond to the plant compounds (Figure 1). Subsequent competitive tests against the isogenic wildtype parent can assess the significance of each gene and compound and may lead to development of a genetic cassette that can be used to promote root colonization by introduced strains. We offer here a preliminary report on attempts to develop signal-responsive mutants of *R. meliloti* and *A. tumefaciens* which can measure the contribution of particular alfalfa compounds to root colonization by these bacteria. Compounds chosen for these studies have all been reported as being released from germinating seeds or roots of alfalfa (Rovira, Harris 1961; Phillips et al. 1992; Koshino et al. 1993).

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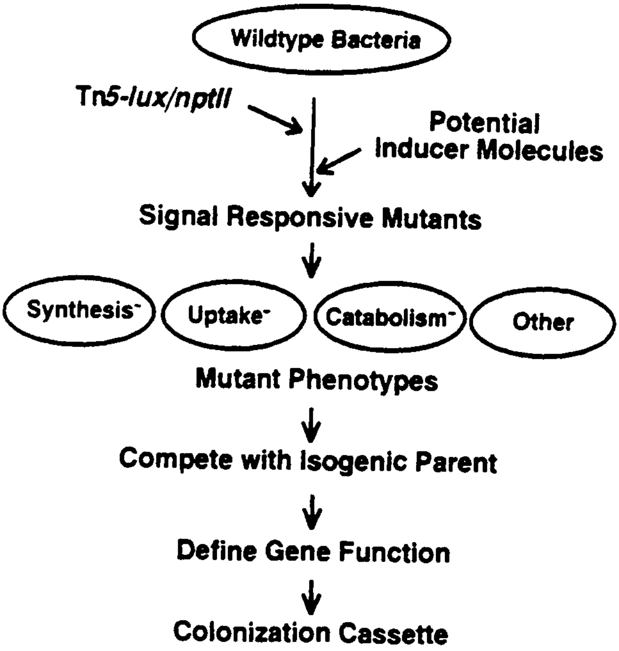
Wildtype Bacteria
Tn5-lux/nptII
Potential Inducer Molecules
Signal Responsive Mutants
Synthesis⁻
Uptake⁻
Catabolism⁻
Other
Mutant Phenotypes
Compete with Isogenic Parent
Define Gene Function
Colonization Cassette

Figure 1. One strategy for finding bacterial genes regulated by plant compounds. Signal-responsive mutants in which a promoterless reporter gene is regulated by the presence of a plant compound can be separated into various physiological phenotypes involving uptake, catabolism, synthesis, and other inducible loci. Genes tagged by the mutagenesis can be recovered for construction of a colonization gene cassette, if they contribute significantly to rhizosphere competence.

Mutants were generated with transposable promoter probes (Tn*5-luxAB* or Tn*5-nptII*) (Wolk et al., 1991; Simon et al., 1989) in *R. meliloti* 1021 (Lim et al. 1993) or *A. tumefaciens* 1D1609, a field isolate from an alfalfa crown gall in the California Imperial Valley. All mutants except At1D1609-I-2 were isolated for their responses (bioluminescence or growth on kanamycin/neomycin) in the presence of potential alfalfa signal compounds (Table 1). Mutant At1D1609-I-1 is induced by both coumestrol and formononetin. At1D1609-I-2 was selected from Tn*5-nptII* mutants for decreased growth relative to the wildtype on coumestrol in the absence of kanamycin or neomycin. This latter isolate may be viewed as a phytoalexin-sensitive mutant which responds with slower growth in the presence of coumestrol. Responses are elicited in the respective mutants by 8 μM biotin, 100 μM stachydrine, 10 μM coumestrol, and 10 μM formonetin.

Plant tests were conducted under microbiologically controlled conditions in Leonard jars (Cunningham et al., 1985). Colony forming units (cfu) were determined in the inoculum at planting and in root rinses 6 days later. For single-strain growth tests a wildtype control was run simultaneously with a comparable initial inoculum. Wildtype and mutant cells were distinguished in competition tests by differing antibiotic resistances.

All tests were repeated at least once with similar results. Data in Table 1 and 2 were collected from many separate experiments, and numerical cfu results for mutants responding to different signal compounds should not be compared.

Mutant bacteria showed various growth (Table 1) and competition (Table 2) phenotypes in the alfalfa rhizosphere, but wildtype Nod, Fix, and Vir phenotypes were not altered. All mutants except Rm1021-B-1 grew well in pure culture on a defined minimal medium. The latter strain required biotin for growth under those conditions and thus qualified as a biotin auxotroph (W.R. Streit, unpublished). While Rm1021-B-2 prospered through repeated transfers on biotin-free minimal medium, it grew no better than the biotin auxotroph in the rhizosphere (Table 1), and both strains were poor competitors against wildtype strain 1021 (Table 2). Complex growth and competition relationships evident in two of the biotin-responsive mutants reported here and three additional mutants resulted from closely linked Tn*5* insertions (W.R. Streit, unpublished). The other biotin-responsive isolate, Rm1021-B-5, is mutated in a biotin-inducible locus which is located in a different EcoR1 fragment than the other five mutants. These data indicate that Moapa 69 alfalfa does not supply enough biotin to support full growth of *R. meliloti*, and that endogenous biotin production is one factor in rhizosphere competence.

Table 1. Growth of individual bacterial strains in the alfalfa rhizosphere. *R. meliloti* (Rm) and *A. tumefaciens* (At) mutants were inoculated on Moapa 69 alfalfa at planting and recovered from roots 6 days later. Phenotypes reflect how mutants interact with the signal compound.

Strain	Signal Compound	Mutant Phenotype	Initial Inoculum	Final Population	Wildtype Growth Difference
			cfu/root		P
Rm1021-B-1	Biotin	Synthesis$^-$	22	474	0.05
Rm1021-B-2	Biotin	Regulatory	35	471	0.05
Rm1021-B-5	Biotin	Inducible	27	1.64 x 10^4	ns
Rm1021-S-10	Stachydrine	Uptake$^-$	319	5.20 x 10^5	ns
Rm1021-S-11	Stachydrine	Catabolism$^-$	99	1.40 x 10^6	ns
At1D1609-I-1	Coumestrol	Inducible	1,730	3.11 x 10^6	ns
At1D1609-I-2	Coumestrol	Sensitive	1,760	2.04 x 10^6	ns

Stachydrine from the germinating alfalfa seed may serve both as an energy source for soil bacteria and as an osmoticum for combating salt stress. Neither Rm1021-S-10 nor Rm1021-S-11 grew on stachydrine as a sole carbon and nitrogen source. The isolates differed, however, in that the former could not use stachydrine to overcome salt stress in pure culture while the latter did (E. Sande, unpublished). These and other results imply that Rm1021-S-10 is incapable of taking up stachydrine, while Rm1021-S-11 is a catabolic mutant. Impaired competitiveness of both mutants against the isogenic parent strain (Table 2) supports the concept that stachydrine from the plant contributes to root colonization by *R. meliloti*.

The isoflavonoid-responsive mutants in *A. tumefaciens* grew well in the rhizosphere in single-strain tests (Table 1), but both grew poorly in competition with the isogenic wildtype parent (Table 2). These isolates were still virulent on alfalfa, and thus it is reasonable to suggest that the chromosomal mutations are located in genes involved with competitive rhizosphere competence (J.D. Palumbo, unpublished).

Table 2. Growth of mutant bacteria in competition with the isogenic wildtype in the rhizosphere of Moapa 69 alfalfa. Bacteria were inoculated at low titers and recovered from the root 6 or 8 days later for *R. meliloti* and *A. tumefaciens*, respectively.

Strain	Inoculum Ratio	Recovered Ratio
	mutant/wildtype	
R. meliloti 1021	1.0	1.00
Rm1021-B-1	5.0	< 0.01*
Rm1021-B-2	5.0	0.02*
Rm1021-B-5	1.0	0.95
Rm1021-S-10	1.0	0.26*
Rm1021-S-11	1.0	0.43*
A. tumefaciens 1D1609	1.0	1.00
At1D1609-I-1	1.0	0.17*
At1D1609-I-2	1.0	0.03*

* Growth of mutant was significantly poorer than wildtype ($P < 0.05$)

The mutated genes in these bacterial strains have been isolated and are being sequenced to test for homologies to previously studied loci. While it is too early to speculate on the relative importance of various genes for root colonization, several facts are evident: First, soil microbes contain genes in addition to *nod*, *nol*, and *vir* which respond to plant compounds. Second, many of these genes affect competitive root colonization. Third, the postulated role of stachydrine as a regulator of rhizosphere microbes is strongly supported with ecological data obtained under controlled conditions.

Results with the biotin mutants suggest that vitamin molecules supplied by plants deserve further examination for their effects on soil bacteria. Relatively little work has been done with B vitamins in the rhizosphere since the initial report of their exudation by legume roots (Rovira, Harris, 1961). It is easy to dismiss vitamins as simple building blocks for microbial protoplasm, but that perfunctory verdict overlooks one important fact: In the rhizosphere, growth equals success on both a temporal and an evolutionary time scale. Any mechanism that bacterial or fungal cells exploit to increase uptake and use of plant-derived vitamins may confer a selective advantage. For example, increasing biotin uptake may help bacterial cells incorporate plant-derived carbon dioxide more effectively. One reaction in which extra biotin and carbon dioxide could help cells grow more rapidly is synthesis of malonyl CoA by acetyl-CoA carboxylase. Enhancing this first committed step in fatty acid synthesis could facilitate membrane formation and cell division.

Mutants described here offer several separate mechanisms for testing eco-physiological hypotheses in the rhizosphere. The coumestrol/formononetin-inducible locus mutated in At1D1609-I-1 presents an opportunity to quantify the importance of those isoflavonoids in controlling an unknown gene required for competitive root colonization, but identifying the actual function of the gene product may be difficult. Stachydrine-inducible mutants Rm1021-S-10 and Rm1021-S-11 show that blocking uptake or catabolism of a molecule creates a very useful tool for dissecting ecological relationships in which one organism supplies that compound to another. More than 400 relatively small organic molecules have been identified from alfalfa by various workers, and any of those molecules that reaches soil microbes potentially could affect root colonization (Phillips, Streit, 1995). The availability of signal-responsive bacterial mutants offers a significant tool for exploring such interactions.

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NODULE DEVELOPMENT IN LEGUMES—THE EARLY STAGES: INVOLVEMENT OF EARLY NODULINS, LECTINS, AND OTHER PROTEINS

HIRSCH, A.M.(1), FANG, Y.(1), BRILL, L.M.(1), WYCOFF, K.L.(2), NINER, B.M.(1), BRANDT, J.P.(1) AND VAN RHIJN P.(1)
(1) Department of Biology, 405 Hilgard Avenue, University of California, Los Angeles, CA 90095-1606 USA
(2) Present address: Plant Biotechnology, 8445 Camino Santa Fe, Suite 102, San Diego, CA 92121

Alfalfa (*Medicago sativa* L.) nodules, like other indeterminate or cylindrical nodules, develop from the *de novo* initiation of cell divisions in the already differentiated cells of the inner root cortex. In contrast, determinate or spherical nodules, like *Lotus* or soybean (*Glycine max* L.) originate from cell divisions in the outer root cortex. In both cases, cell divisions are initiated soon after inoculation with *(Brady)Rhizobium* or after treatment with Nod factor. In indeterminate nodules, a nodule primordium develops within the inner root cortex and a nodule meristem is secondarily established along its periphery. The nodule meristem serves as the source of the various tissues in the nodule. In determinate nodules, the nodule primordia cells divide, and then differentiate into their final state, thereby forming the nodule tissues.

For many years, two important ideas have influenced our thoughts on nodule development. One is the concept that plant hormones play an important role in nodulation. As a step to demonstrate the usefulness of this notion, we have shown that several of the stages involved in indeterminate nodule formation can be induced by altering the root's endogenous hormone balance. Cell divisions and early nodulin (ENOD) gene expression can be triggered by treating the root with auxin transport inhibitors (Hirsch, et al., 1989; Scheres et al., 1992) or cytokinins (Fang et al., 1995).

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The second concept that has greatly influenced nodule development, and particularly host-*Rhizobium* recognition, deals with the involvement of lectins in nodulation. Bohlool and Schmidt (1974), in the lectin recognition hypothesis, proposed that the specific binding of a lectin to rhizobial polysaccharides enabled a compatible *Rhizobium* to recognize its host. We are studying the involvement of lectins in nodulation by transferring the soybean seed lectin gene into *Lotus* and alfalfa, and by characterizing lectin genes in alfalfa. In addition, we have constructed lectin-antisense plants of alfalfa.

Msenod40 expression is triggered by cytokinin application.

The MsENOD40 gene product has been proposed to function as an RNA (Asad et al., 1994; Crespi et al., 1994). The RNA has no AUG translational start site; there are only very short ORF's that begin with a methionine. Its spatial pattern of expression suggests that the gene product functions in cells that are dividing or are poised to divide. MsENOD40 transcripts are detected in the meristem and also in the pericycle of mature alfalfa nodules (Fig. 1). In addition, transcripts are found in the procambium of the stem, the apical meristem of lateral roots, and in leaf primordia.

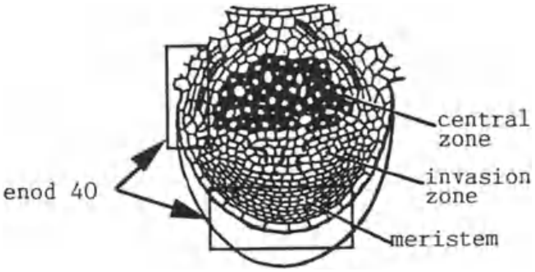

Fig. 1. Mature nodule of alfalfa. *Msenod40* is expressed in the nodule meristem and the pericycle (boxed).

Among the different phytohormones, we have determined that the application of cytokinin or auxin transport inhibitors results in *Msenod40* expression (Fang et al., 1995).

When alfalfa roots are inoculated with a Nod⁻ *Rhizobium meliloti* transconjugant carrying the *Agrobacterium tumefaciens tzs* (*trans*-zeatin synthase) gene, small, uninfected nodules are formed (Cooper and Long, 1994). We find that these small nodules contain MsENOD40 transcripts and they are localized to the meristematic edges. Moreover, when we treat the non-nodulating alfalfa mutant, MN1008, with these transconjugants, nodule-like structures that resemble aborted nodules, form; these contain MsENOD40 transcripts. Cytokinin application to roots of MN1008 does not induce cell division, but rather causes a change in the structure of the outer cortical cells. They become expanded, and the cortical cell cytoplasms reorient so that the nucleus moves from a peripheral to central position. These results indicate that a change in hormone levels or sensitivities induces cytoplasmic reorientation, cell divisions, and MsENOD40 gene expression in alfalfa roots.

Studies on alfalfa lectin.

We have isolated and characterized a full-length alfalfa lectin gene that encodes a protein which is 92% identical to MtLEC1 described early by Bauchrowitz et al. (1992) (Brill et al., 1995). We have also isolated and partially sequenced *Mslec2*, which was identified by its homology to *Mtlec2* (Bauchrowitz et al.,1992). Unlike *Mtlec2*, which is likely to be a pseudogene, *Mslec2* is expressed. Using a small region of *Mslec2* (β-region; positions 321-721), we determined that this gene is expressed in stems, flowers, and nodules. Although transcripts were detected in roots using medium stringency washes (0.5 X SSPE, 55° C) , the signal disappeared after high stringency washes (0.1 X SSPE, 65° C) (Fig. 2).

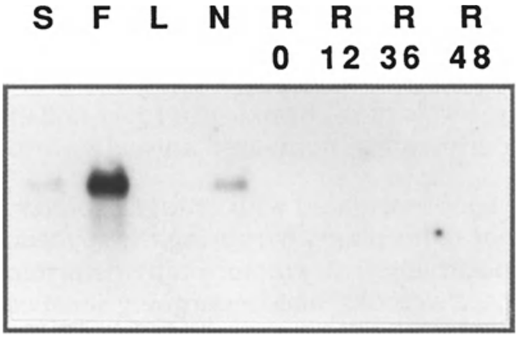

Fig. 2. Northern blot of RNA (10 µ g/lane) isolated from different alfalfa tissues (S, stem; F, flower; L, leaf; N, nodule; R0, root 0 hours after inoculation with *Rhizobium meliloti;* R12, 12 hours post-inoculation; R36, 36 hours post-inoculation; R 48, 48 hours post-inoculation.

We were able to use small regions of the *Mslec1* (γ-region; positions 1280-1700)and *Mslec2* (β-region)to differentiate between the two different genes, and cloned these in antisense orientation into plant transformation vectors to introduce into *M. sativa* cv. Regen. Some obvious differences were apparent between the vector controls, γ-antisense plants, and β-antisense plants. The antisense plants produced fewer embryos overall than the control plants although the time of embryogenesis was approximately the same among the three lines. In addition, the antisense plants, especially the β-antisense plants, exhibited a tendency to form teratomas. These results are summarized in Table 1.

Table 1. Summary of Characteristics of Lectin Antisense Alfalfas

γ (*MsLec1*)	β (*MsLec2*)
▤severe defects in somatic embryogenesis ▤defects in root growth and structure ▤moderate teratoma formation	▤moderate defects in somatic embryogenesis ▤defects in root growth and structure ▤moderate teratoma formation

Studies on the soybean lectin.

We transferred the soybean lectin gene under the CaMV 35S promoter and the control plasmid pBI121 (Jefferson et al., 1987) into *A. tumefaciens,* LBA4404, which was used to transform alfalfa leaves. *Lotus* was transformed by the method of Szabados et al., (1990). Using a soybean seed lectin antibody, we determined that the transformed plants produced varying levels of soybean lectin (data not shown). All plants, controls and experimentals, nodulated normally with *R. loti.*

Transgenic plants were spot-inoculated with *Bradyrhizobium japonicum* USDA110. We found that *Lotus* plants harboring the soybean seed lectin gene responded positively to *B. japonicum* by deforming root hairs, developing shepherd's crooks, and undergoing some cortical cell divisions. These small bumps, however, were uninfected. The results for *Lotus* are summarized in Table 2.

Table 2. The effect of spot-inoculating *Bradyrhizobium japonicum* USDA110 on transgenic plants containing the soybean lectin gene.

Plant	**Had**	**Hac**	**Ccd**
Lotus /lecGR	+	+	+ (6/49)
Lotus/lecWest	±	-	-
Lotus /lec3	+	+	+ (4/36)
Lotus/lec7	+	+	+ (2/17)
Lotus/control	±	-	-

In contrast, alfalfa plants did not undergo either root hair curling or cortical cell divisions in response to *B. japonicum.*

Conclusions.

It is premature to state exactly how hormones or lectins are involved in nodulation. However, we have shown that some early nodulin genes are induced by perturbations in the plants endogenous hormone balance brought about the application of auxin transport inhibitors or cytokinin. The fact that these events occur in a non-nodulating mutant suggests that this change is independent and downstream of Nod factor. In addition, this hormone imbalance is correlated with a cytoplasmic reorientation that may involve the plant cytoskeleton. How the change in the cytoskeleton is mediated is unknown. Perhaps lectins as extracellular or plasma membrane-bound proteins (Kijne et al., 1994) may be part of the signal transduction pathway. However, their precise role in nodulation is still unclear. The lectin antisense plants may allow us to test more directly the involvement of this protein in nodulation.

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SIGNAL EXCHANGE DURING SOYBEAN NODULATION BY *BRADYRHIZOBIUM JAPONICUM.*

STACEY, G.(1), MINAMI, E.(1,2), KOUCHI, H.(2), COHN, J.R.(1), CARLSON, R.W.(3)
(1)Center for Legume Recearch, University of Tennessee, Knoxville, TN 37996-0845, USA; (2)Dept. of Cell Biology and Dept. of Applied Physiology, National Institute of Agrobiological Resources, Tsukuba, Ibaraki, 305, Japan; (3)Complex Carbohydrate Research Center, University of Georgia, 220 Riverbend Rd., Athens, GA 30602-4712, USA.

Rhizobium, *Azorhizobium* and *Bradyrhizobium* species infect leguminous plants and induce root organogenesis resulting in a nodule in which the symbionts reside. Legume nodules can be classified into 2 types based on their ontogeny; that is, indeterminate (e.g., alfalfa) and determinate (e.g., soybean). Indeterminate nodules develop from initial divisions of inner cortical root cells and have a persistent meristem. Determinate nodules form from the initial division of sub-epidermal cortical cells and meristematic activity is limited only to the early stages of nodule development. Since the ontogeny of determinate and indeterminate nodules differs significantly, it can be expected that the signal pathways involved in these two systems likely also differ.

The rhizobial nodulation (*nod*) genes are essential for nodule formation and their transcription is induced in response to plant-produced flavonoids (Peters, Verma, 1990). It is now known that many of the *nod* genes encode enzymes involved in the synthesis of substituted lipochitooligosaccharide (LCO) molecules (Carlson et al. 1994). These molecules serve as a signal to the plant and induce the early steps in nodule organogenesis. The nodulation signals consist of a chitin oligomer (3-5 residues) modified by a N-acyl group at the non-reducing terminus and by a variety of substituents on the terminal, reducing sugar.

B. japonicum nodulation signals: In *Bradyrhizobium japonicum*, the primary nodulation signal is a chitin pentamer N-acylated with vaccenic acid and modified on the terminal, reducing sugar by 2-O-methylfucose [i.e. NodBj-V (C18:1 Δ11, MeFuc); Sanjuan et al. 1992; Carlson et al. 1993]. However, in addition to this primary signal, we have identified 15 additional LCO molecules produced by *B. japonicum* or *B. elkanii* wildtype or mutant strains (Carlson et al. 1993; Luka et al. 1993; Stacey et al. 1994). These molecules differ in the length of the chitin backbone, specific substituents, and/or amount produced.

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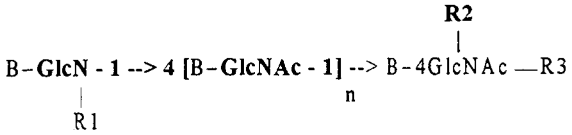

$$\underset{\displaystyle R1}{\text{B-GlcN}} - 1 \text{-->} 4\ [\text{B-GlcNAc} - 1]_{n} \text{-->}\ \text{B-4GlcNAc}\overset{\displaystyle R2}{} \text{—R3}$$

Table 1. SYNTHETIC NOD SIGNALS

LCO	R1	R2	R3	n	HAD	NOI
1	C18:1(11)	2-O-MEFU	H	3	+	+
2	C18:1(9)	2-O-MEFU	H	3	+	+
3	C16:0	2-O-MEFU	H	3	+	+
4	C16:0	H	H	2	+	+
5	C16:0	SO3	H	2	-	-
6	C16:2	SO3	H	2	-	-
7	C16:1	SO3	H	2	-	-
8	C16:1	SO3	H	2	-	-
9	C16:0	2-O-MEFU	H	2	-	-
10	C18:1(11)	2-O-MEFU	H	2	-	-
11	C18:1(11)	FUC	R-GRO	2	-	-
12	C18:1(11)	FUC	S-GRO	2	-	-
13	C18:1(9)	FUC	R-GRO	2	-	-
14	C18:1(9)	FUC	S-GRO	2	-	-

Structure/function studies of nodulation signals: Inoculation of legume roots with purified nod signals elicits on the compatible host physiological responses, such as root hair deformation (HAD) and root cortical cell division (NOI) (Spaink 1994), that mimic those seen after bacterial inoculation. To better understand the role of various LCO structural variants in the nodulation process, we undertook a detailed structure/function study of the various lipo-chitin signals produced by *B. japonicum*. We were aided in this research by a variety of chemically synthesized lipo-chitin molecules (Table 1; Ikeshita et al., 1994, 1995). Initially, our work focused on bioassays of different LCO molecules to measure their ability to induce a HAD or NOI response on *G. soja* roots (Table 1, Stokkermans et al. 1995). Of the various structural analogues that were tested, only 4 LCO were active. In order of activity, LCO-V(C18:1Δ11,mefuc), LCO-V(C18:1Δ9,mefuc), LCO-V(C16:0,mefuc), and LCO-IV(C16:0) were found to induce both a HAD and NOI response. The first of these compounds is identical in structure to the most abundant natural *B. japonicum* nod signal. This work showed conclusively that the specificity of the *B. japonicum* nodulation signals is determined by an interaction between the length of the chitin oligomer and various substitutions to the chitin chain. Since it is difficult to rule out the possibility of unsuspected contaminants in natural LCO preparations, another important finding of these studies was that synthetic LCO molecules possess identical biological activities to those purified from *B. japonicum* cultures.

Induction of nodulin gene expression in response to lipo-chitin nodulation signals: A. ENOD40 expression. Nodule organogenesis is genetically controlled by the host plant. One aspect of this process is the induction of genes specific to nodules, so-called nodulins (Nap, Bisseling, 1990). Nodulins are classified into early and late nodulins according to their time of activation subsequent to bacterial inoculation. Of those nodulins known to be induced rapidly upon inoculation, ENOD40 (Yang et al., 1993) (also called GmN36, Kouchi, Hata, 1993) was shown to be expressed very early during soybean nodule initiation. Therefore, ENOD40 expression can be used as an early molecular marker for the initiation of nodule primordia. In situ hybridization analysis showed that ENOD40 mRNA was abundant in root pericycle cells before the appearance of infection threads. These same studies detected the expression of ENOD40 in the pericycle cells surrounding vascular strands in mature nodules.

In order to continue our studies of the structure/ function properties of various LCO molecules, we chose to examine the expression of ENOD40 after addition of nod signals. Initial accumulation of ENOD40 mRNA was detected, after amplification by reverse transcription and polymerase chain reaction, within 6h of bacterial inoculation or addition of active LCO. This initial burst of ENOD40 mRNA expression appears to peak at approximately 40 h after LCO addition. A second rise in ENOD40 mRNA expression is seen by 4d after LCO addition, but only with those LCO known to induce a HAD or NOI response (see above). A suprising finding from these studies was that LCO found to be inactive in inducing a HAD or NOI response would induce ENOD40 mRNA accumulation when assayed 40h after inoculation. Indeed, roots inoculated with a non-acylated, un-substituted chitin pentamer expressed elevated levels of ENOD40 mRNA. However, chitosan oligomers (i.e., non-acetylated chitin) did not possess this activity. The difference in the plant response to molecules such as LCO-V(C18:1Δ11,mefuc) and chitin pentamer was that only the former compound was able to elicit the second burst of ENOD40 mRNA expression beginning at approximately 4d. Therefore, the initial plant response to LCO is rapid and somewhat non-specific. In situ hybridization studies indicated that this rapid accumulation of ENOD40 mRNA occurs in the pericycle. The second rise in ENOD40 mRNA expression seen at 4-6d requires a specific LCO structure and coincides in specificity to that seen previously for a HAD or NOI response. This later accumulation of ENOD40 mRNA occurs both in the pericycle and dividing subepidermal cortical cells.

B. ENOD2 and ENOD55 expression: Our hypothesis to explain the variety of LCO molecules produced by *B. japonicum* has been that these mixtures arise from two factors; 1. some of the factors isolated are biosynthetic intermediates and 2. a diversity of nod signals is required for nodulation of a broad range of host plants. However, a third reason for the variety of LCO produced by *B. japonicum* has become apparent from our studies of ENOD2 and ENOD55 expression in *G. soja* roots. ENOD2 is an early nodulin gene that encodes a hydroxyproline rich, cell wall protein (van de Wiel et al., 1990). ENOD2 is expressed rapidly upon bacterial inoculation. ENOD55 is also expressed rapidly and has been proposed to be a cell wall protein. ENOD55 expression has been localized to infected cells at an early stage of nodule development (Kouchi, Hata, 1993). Since both ENOD2 and ENOD55 are early nodulins, we were puzzled by the inability to induce their expression by the addition of LCO known to induce a HAD or NOI response. Since individual lipo-chitin nodulation signals did not induce ENOD2 or ENOD55 mRNA expression, we tested crude butanol extracts of *B. japonicum* cultures induced for *nod* gene expression. This is the starting material for our purification of LCO. The results indicated that this crude mixture of material had the ability to induced both ENOD2 and ENOD55 expression. Since we had in our possession various synthetic and naturally purified LCO, we began to test a variety of mixtures for their ability to induce these early nodulins. After a number of experiments, we found that a mixture containing any two of the LCO known to induce a HAD or NOI response was sufficient to induce ENOD2 or ENOD55 expression. However, it was essential that at least two structurally distinct nodulation signals were added. Considering our previous results showing that non-specific LCO could induce ENOD40 expression, we tested these LCO in various combinations. The results indicated that a mixture containing a LCO capable of inducing a HAD or NOI response (e.g., LCO-V(C18:1Δ11,mefuc) and one lacking this activity (e.g., chitin pentamer) was sufficient to induce ENOD2 and ENOD55 expression. Indeed, a mixture of the primary nodulation signal of *B. japonicum* (i.e., LCO-V(C18:1Δ11,mefuc) and that from *R. meliloti* (i.e., LCO-IV(16:2Δ2Δ9,SO_3) was sufficient to elicit expression of these nodulins.

Model for nod signal reception. The discovery that at least two structurally distinct nodulation signals are required to induce ENOD2 and ENOD55 expression and to mimic with fidelity bacterial inoculation puts specific limits on possible models for nod signal action. Our results have led us to formulate the model shown in Figure 1 which has two major features:

1. Specific chemical substituents on the lipo-chitin nod signals, previously thought to be essential for biological activity, are not required for the rapid induction of some soybean early nodulin genes. However, these specific substituents are required for later events. These data lead to us to postulate the existence of two receptors that interact with chitin and lipo-chitin signals. Only one of these receptors (i.e., NF_S) has a high degree of specificity with regard to chitin oligomer recognition.

2. The induction of some early nodulin genes requires the synergistic action of two, structurally distinct chitin Nod signals. The data lead us to propose that one signal must interact with the specific receptor NF_S, while an additional signal interacts with a less specific receptor NF_G.

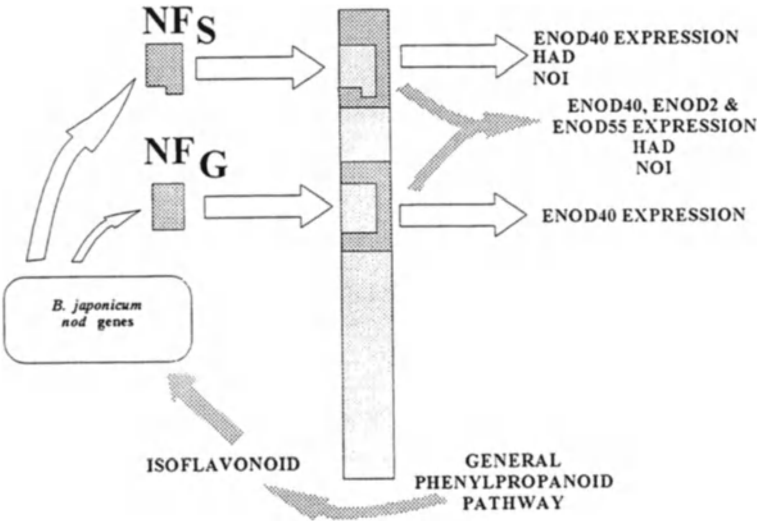

Figure 1. Model of signal transduction pathways involved in soybean nodulation. NF_G= non-specific chitin signal. NF_S= specific chitin signal.

In some respects our model resembles that proposed by Ardourel et al. (1994) who also suggested that two receptors were involved in *R. meliloti* nod signal recognition by alfalfa. In the model of Ardourel et al. (1994), a 'signaling receptor' is responsible for recognition of the non-specific nod signals and results in the induction of cell wall tip growth and cortical cell division. A more specific recognition event at an 'entry receptor' is required for the initiation of an infection thread, marked root hair curling and induction of a true nodule primordium. In the case of soybean, we propose that a general 'signaling receptor' (NF_G) recognizes chitin pentamer and other non-specific LCO allowing for the rapid induction of ENOD40 expression. Unlike in the case of Ardourel et al. (1994), cortical cell division does not accompany this response. It is quite possible that a general chitin receptor may be involved in this initial response in soybean. A more specific 'entry receptor' (NF_S) may be necessary in soybean for recognition of those LCO capable of inducing a HAD and NOI response. However, the response mediated by this 'entry receptor' in alfalfa and soybean appear somewhat different. Specifically, the induction of cortical cell division in soybean appears to require a more specific recognition event than is apparent from studies in alfalfa. This may reflect the differences in location of the initial cell divisions that occur during the ontogeny of determinate (i.e., subepidermal cortical cells) vs. indeterminate nodules (i.e., inner cortical cells).

In addition to the spatial discrimination between nod signal responses in roots discussed by Ardourel et al. (1994), our results show a temporal difference in response. With regard to ENOD40 expression, nod signal specificity was manifested only in a secondary burst of expression at 4-6 days post inoculation. This coincides with the development of the nodule primordium. A surprising finding of the study of Stokkermans et al. (1995) was the strict correlation between the HAD and NOI response in *G. soja* roots to various nod signals. Such a strict correlation was not found previously in studies with *Vicia sativa* which forms indeterminate nodules (Spaink et al., 1991; van Brussel et al., 1992). Therefore, the signaling in soybean appears quite different from that in *Vicia* and, perhaps, all plants forming indeterminate nodules.

The model shown in Figure 1 is speculative but consistent with our recent results and provides us with an experimental system on which to base specific and testable hypotheses.

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THE INFLUENCE OF NOD METABOLITES ON ROOT HAIR CURLING AND DEFORMATION

ABE, M., YAMASHITA, H., HIGASHI, S., AND UCHIUMI, T.
Department of Biology, Faculty of Science,
Kagoshima Univesity, Kagoshima 890, JAPAN

The symbiotic communications between *Rhizobium* and the conformable host plant have been examined on exchange of their signal molecules

The host plant signal molecule(s) (flavonoids) is exuded to the rhizosphere and induces *nod* genes expression of*Rhizobium*. In this work, we isolated the Nod metabolites from *Rhizobium leguminosarum* bv. *trifolii* by flavonoid (Apigenin) addition in the culture medium. Strain H1(pC4S8), carrying 17.75 kb *nod* genes of strain 4S (wild type) pSym cured strain H1, was used in this experiment. This strain has phenotypes Hac+, Had+, Inf+, Nod+ and Fix$^-$.The Nod metabolite(s) was isolated by n-BuOH-extraction from the periplasma containing materials of bacterial cell and culture supernatant. The Nod metabolite(s) was applied to the clover seedlings (root length, 5 mm) and incubated at 25° C in the dark. After 3 days incubation, the root hair deformation and curling was observed by light microscope. The levels of root hair deformation and curling were classified into three types; **A**-type, curling and branching with swelling on the short root hair cell, **B**-type, curling and branching on the root hair cell without swelling, **C**-type, no deformation and/or no specific deformation.The marked deformation (**A**-type and **B**-type), which is expected as strongly related with *Rhizobium* and Legume symbiosis, was caused by the addition of the extracts from bacterial periplasma. It is supposed that the synthesized Nod metabolite(s) of *R. l.* bv. *trifolii* as responding to plant signal flavonoid will be kept in bacterial periplasma than exuded to the extracellular fraction. The attractive molecule(s) synthesized in periplasma may attach to only limited region of root hair cell at where bacterial cell attach, and induce to root hair cell wall's irregular growth. ODS-HPLC and Silica Gel-HPTLC analysis is performed to isolate the Hac and Had factor from Nod metabolites of strain H1(pC4S8), at least 2 peaks of HPLC elution after extraction from HPTLC, caused strong **A**- and **B**-type deformation on the clover root hairs.

Rhizobium etli LIPO-OLIGOSACCHARIDE SIGNALS. PURIFICATION AND BIOLOGICAL ACTIVITY.

CARDENAS, L.[1], THOMAS-OATES, J.E.[2], LOPEZ, I.[2],
SPAINK, H.P.[2], DOMINGUEZ, J.[1] AND QUINTO, C.[1]
[1]Dept. Biologia, Molecular de Plantas, Inst. de Biotecnologia, niv. Nacional Autonoma de Mexico, Cuernavaca, Morelos, Mexico.
[2]Clusius Lab, Inst. Molecular Plant Sciences, Wassenaarseweg 64,
2333 AL Leiden, 2300 RA Leiden, The Netherlands

The interaction between bacteria belonging to the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* and their leguminous plants culminates in the formation of nitrogen-fixing root nodules. One of the first steps of this complex process of nodule formation involve a mutual recognition of signal molecules produced by plant and bacteria. Flavonoid signal molecules produced by plant are recognized by the guest bacteria, specifically by the regulatory NodD protein. As a result of this recognition, the NodD protein activates the transcription of the *nod* and *nol* genes and subsequently the bacteria produce signal molecules which are specifically recognized by the host plant. The signal molecules (also called Nod metabolites) from several rhizobial strains have been identified and they all appear to consist of an acylated chitin oligosaccharide which can contain strain-specific modifications. Herein we report the isolation and structural elucidation of the lipo-chitin oligosaccharide (LCO) signal molecules produced by *Rhizobium etli*, a symbiont of *Phaseolus vulgaris*. They belong to the same LCO family as described for Nod factors from other rhizobial species, having an acetyl fucose on the reducing end, a *N*-methyl group on the same nitrogen atom to which the fatty acyl is linked and a carbomyl group. In this report, we demonstrate that LOS purified from *R. etli* are able to induce nodule-like structures on *P. vulgaris* without the presence of the bacteria. These results and the characteristic structural features of the *R. etli* LCO are presented and discussed.

COMPETITIVENESS OF *RHIZOBIUM LEGUMINOSARUM* STRAINS IN THE CONNECTION WITH NOD FACTOR PRODUCTION.

KRAVCHENKO[1] L.V., MAKAROVA[1]N.M., OVTSYNA[1] A.O.,
KULIKOVA[1] O.A., SPAINK[2] H.P., LUGTENBERG[2] B.J.J.,
TIKHONOVICH[1] I.A.
[1] All - Russia Research Institute for Agricultural Microbiology,
St.Petersburg, 189620, Russia
[2] Institute of Molecular Plant Sciences Leiden University, NL-2333 AL
Leiden, The Netherlands

Rhizobium leguminosarum strain A1 isolated from North - Western soils of Russia (Chetkova, Tikhonovich, 1986) forms effective and highly specific symbiosis with *sym2sym2* - carrying peas. This strain accumulates stably in soil without loosing infective ability. Induction of A1 with naringenin led to the increasing of infective and competitive ability, nitrogenase activity of the strain on some pea lines. Strain A1 is not suppressed by 4 highly competitive *R.leg.* strains from RIAM collection which don't nodulate the Afghanistan peas. Co-inoculation of Aghanistan pea by A1 with each of these strains resulted in formation of functional nodules occupied only by strain A1. In co-inoculation experiments with strain TOM (Lie, 1978) strain A1 did not suppress nodulation by TOM. Triple inoculation (A1, TOM and cnb$^+$ strain CIAM1026) resulted in the formation of nodules 100% infected by strain A1. Since competitive ability of the strain depends on the Nod metabolite production we studied structure of LOS produced by strain A1. The HPLC and mass - spectrometry analysis revealed that strain A1 produces a family of differently substituted Nod factors. Among them there are molecules common for standard *R.leg.bv.viceae* and double acetylated factor analogous to those produced by strain TOM (Firmin et al., 1993). However, besides them strain A1 synthesizes molecules with carbamoyl group and with unusual fatty acids - C18:2 and C18:3. Unlike the strain TOM strain A1 is characterized by high production of Nod metabolites. This fact is not consistent with the proposed role of significance of small Nod factor amounts for nodulation of Afghanistan peas (Downie et al., 1994.). We suppose that strain A1 is not recognized by the plant defense system due to new specific modifications of Nod factors. It is also possible that enhanced LOS production contributes to high competitive ability of the strain A1.

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PHYSIOLOGICALLY ACTIVE SUBSTANCES OF RED CLOVER AND THEIR ROLE IN SYMBIOTIC NITROGEN FIXATION.

KUZNETSOVA, L.G.[1], KOSOUROV, S.N.[2], KEFELI, V.I.[3]

(1), (3) Institute of Soil Science and Photosynthesis, Russian Academy of Sciences, Pushchino, Moscow Region 142292, Russia.

(2) Pushchino State University, Moscow Region, 142292, Russia.

Red clover plants were grown under different nitrogen concentrations. The content and the activity of physiologically active substances (PHAS) changed sharply both at high nitrogen concentration and at the absence of nitrogen (NO_3^-). Three groups of PHAS have been extracted, two of them had rather high cytokinin activities. The first group, including substances with absorption maximum at 264 nm and no absorption shift, was observed in alkaline pH value. It was suggested that these hormones are cytokinin-sugar-conjugates. The content of these substances was low in plants grown continuously at the high nitrogen concentration and it increased with a decrease of nitrogen supply. However, the cytokinin activity changed reciprocally, i.e. it was high at 60 mM NO_3^- and low without any nitrates. The second group contained unknown substances which demonstrated blue fluorescence under UV-light at 254 nm and an absorption shift in the red spectrum at 10 nm was observed. Their concentration increased 2 to 3-fold in plants grown in nitrate-free solutions. The highest content of the substances in the second group was noted in plants with a high level of nitrogen fixation. The substances of both groups were mainly the leaves and this influenced on sink-source relation. The third group consisted of alkaline indole derivatives. These compounds having weak auxin activity were discovered only in root tissues. We made a conclusion that substances of the first group and the second one are important for symbiotic nitrogen fixation by red clover.

ENHANCED ADSORPTION TO SOYBEAN ROOTS, INFECTIVITY, AND COMPETITION FOR NODULATION, OF *Bradyrhizobium japonicum* PRETREATED WITH SOYBEAN LECTIN

LODEIRO, A. R.; FAVELUKES, G.
Instituto de Bioquímica y Biología Molecular, Facultad de Ciencias
Exactas, Universidad Nacional de La Plata, 1900-La Plata, Argentina.

The host legume lectin (from root exudates, also found in seeds), is involved in rhizobial preinfection, infection, and symbiotic specificity (see Wall, Favelukes, 1991 for a review). Here we show that *Bradyrhizobium japonicum* Bj 3001 (spectinomycin-resistant derivative of USDA 110) incubated in Fåhraeus solution in the presence of soybean seed lectin (SBL) or soybean seed extract (SSE), acquires an increased ability for adsorption to soybean roots, as well as infectivity, and competitiveness for nodule occupation.

Rhizobial adsorption to roots (assayed as previously described, see ref. above), was stimulated by pretreatment with SBL and by SSE. The stimulatory activity in SSE appeared to depend on the presence of an SBL-like lectin as indicated by its elimination by passing through an ε-aminocaproyl-N-acetyl-ß-D-galactosamine-agarose affinity column, or its inhibition by the SBL hapten 0.43 mM N-acetyl-D-galactosamine (GalNAc). Direct adsorption of Bj 3001 to soybean roots was not inhibited by GalNAc. The lack of activity of the common bean lectin (PHA) or alfalfa agglutinin suggest that the effect is symbiont-specific. Full stimulation was obtained with 10^5 SBL molecules per rhizobia, while a half-maximal stimulation was produced by 300 molecules. These effects required a preincubation of at least 12 to 24 h, while shorter treatments were minimally effective.

Same as reported previously by Halverson, Stacey, 1986, we observed that the delay of 11 h in the initiation of infections with the control rhizobia ($6x10^4$ bacteria per plant) was reduced to only 6 h by prior treatment with SBL, indicating an increased infectivity.

Preincubation of Bj 3001 with either SBL or SSE increased its competitive ability for nodulation. In soybeans inoculated with 1:1 competitor ratios, nodule occupancy by the treated strain was 8 to 20% higher compared with a control-treated, streptomycin-resistant isogenic strain.

These results suggest that, by interacting specifically with the free-living *B. japonicum* early in preinfection, the soybean lectin plays a role in the process of adsorption of the rhizobia to soybean roots, and its effects extend to later stages of symbiosis, favoring infection and competitiveness.

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Wall LG, Favelukes G (1991) J.Bacteriol. **173**:3492-99

NODULATION FAILURE DUE TO EARLY PEROXIDASE RESPONSE: A CONSEQUENCE OF INCORRECT SIGNALLING?

Margreet H. de Boer, Louise F. Roddam and Michael A. Djordjevic. Plant Microbe Interactions Group, Research School of Biological Sciences, Australian National University, GPO Box 475, ACT 2601, Canberra, Australia.

Rhizobium leguminosarum biovar *trifolii* strain ANU794 nodulates a variety of subterranean clovers but is unable to form nodules on *Trifolium subterraneum* cv. Woogenellup. This nodulation failure is due to an inhibition of infection thread elongation within either the root hairs or the root outer cortical cells. Nodule primordia were induced but failed to develop. No signs of hypersensitive cell death were seen (de Boer and Djordjevic 1995). We have demonstrated that a localised infection specific increase in peroxidase activity occurs in the root hair in response to infection of cv. Woogenellup with strain ANU794. *In situ* DAB (diaminobenzidine) staining revealed strain ANU794 specific peroxidase activity at sites of infection thread initiation, in the folds of tightly curled root hairs and on the surface of the root hair cells. This is correlated with a significant increase in peroxidase activity in the cell wall fraction of strain ANU794 inoculated Woogenellup roots within 24 hours, which is further enhanced after 72 hours. Electrophoretic separation of cell wall peroxidases by IEF was used to show an increase in an highly acidic peroxidase isozyme. Consistent with these results, immunochemical localisation of peroxidases showed that the infection threads induced by strain ANU794 were strongly labelled. In contrast, the nodulating strain ANU843 caused a significant decrease in cell wall bound peroxidases after 24 hours compared to the uninoculated control. This was consistent with a reduced DAB staining at the root hair level in comparison to strain ANU794 and a lower level of the acidic peroxidase, suggesting that this reduction of peroxidase activity may be a prerequisite for normal infection.

Strain ANU794 was shown to produce Nod factors with different physiochemical properties to the factors induced by strain ANU843. In addition, 3 new Tn5 induced mutants of strain ANU794 were isolated which showed a significantly increased nodulation on cv. Woogenellup. The patterns of Nod factors produced by these mutants was altered suggesting that the Tn5-interrupted genes are involved in the production or excretion of the Nod factors.

We hypothesise that the Nod factors produced by strain ANU794 result in the initial increase in peroxidase activity on cv. Woogenellup. The new nodulating mutants are useful tools in further examining this hypothesis.

de Boer MH and Djordjevic MA (1995) Protoplasma 185:58-71.

FUNCTIONS OF NOD PROTEINS IN *AZORHIZOBIUM CAULINODANS*

MERGAERT P. (1), D'HAEZE W. (1), GEELEN D. (1), PROMÉ, D. (2), VAN MONTAGU M. (1), PROMÉ, J.C. (2), HOLSTERS M. (1)
(1) Laboratorium voor Genetica, Universiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium; (2) Laboratoire de Pharmacologie et de Toxicologie Fondamentales, CNRS, 205 route de Narbonne, F-31077 Toulouse Cedex, France.

In *Azorhizobium caulinodans* strain ORS571, the production of Nod factors, signals that induce nodule formation on the host plant *Sesbania rostrata*, is dependent on an operon containing the genes *nodABCSUIJZorf9*. These Nod factors are lipo-chito-oligosaccharides modified, on the reducing end, with an arabinosyl branch and, on the non-reducing end, with a O-carbamoyl and N-methyl group. By *in vitro* and *in vivo* studies using ORS571 and *Escherichia coli* expressing *nodABCS* or different combinations of these genes, Nod factor intermediates were purified and their structures were determined with mass spectrometry. Based on these results, the Nod factor biosynthetic pathway was partially resolved.

The NodC protein synthesises *in vitro* and *in vivo* chitooligosaccharides with two to five GlcNAc units. After the synthesis by NodC is completed, the chitooligosaccharides are deacetylated at the non-reducing end by the NodB protein. The NodS protein is a *S*-adenosyl-L-methionine-dependent *N*-methyltransferase and uses chitooligosaccharides deacetylated at the non-reducing end (the products of NodC and NodB) as substrates. *E. coli* cells containing NodABCS produce besides the N-methylated, deacetylated chitooligosaccharides also chitopentasaccharides acylated at the non-reducing end with fatty acids ranging from C14 to C18. This suggests that NodA is an acyltransferase performing the last step in the synthesis of the lipo-chitooligosaccharide core of Nod factors.

Pulse chase experiments show that the N-methylated, deacetylated chitooligosaccharides made in cells expressing *nodABCS* are not the true substrates for the acyl transfer step. This suggests that the Nod proteins act as a complex and that intermediates, released from the complex cannot be further modified to the lipo-chitooligosaccharides. Alternatively, the lipo-chitooligosaccharide synthesis can take place on an aglycone anchor. In this case, this anchor is probably not undecaprenyl phosphate as *in vivo* and *in vitro* tunicamycin and bacitracin have no effect on the synthesis of chitooligosaccharides and lipo-chitooligosaccharides.

CHARACTERIZATION OF A *MEDICAGO* ENZYME WITH LIPO-CHITOOLIGOSACCHARIDASE ACTIVITY

STAEHELIN C.[1], SCHULTZE M.[1], KONDOROSI E.[1], AND
KONDOROSI A.[1,2]
[1]Institut des Sciences Vegetales, CNRS, F-91198 Gif-sur-Yvette, France
[2]Institut of Genetics, Biolgoical Resarch Center, Hungarian Academy
of Sciences, P.O. Box 521, H-6701 Szeged, Hungary

Hydrolases from the host plant are able to cleave and inactivate nodulation (Nod) factors, the bacterial lipo-chitooligosaccharide signals of *Rhizobium*. Hydrolytic activities were distinguished by their different cleavage specificities using different *R. meliloti* Nod factors and their derivatives as substrate. Enzymes from *Medicago* root exudates were fractionated by different chromatographic methods. Lectin affinity chromatography resulted in the separation of two hydrolytic activities with different cleavage specificity. A concanavalin A-binding glycoprotein was able to cleave all tested Nod factors having an oligosaccharide chain length of four or five residues, whereas the acylated trimer NodRm-III was not a substrate for the enzyme. This hydrolase released from the Nod factors only the acylated dimers, NodRm-II and NodRm-II(Ac) from the *O*-acetylated derivatives. The *in situ* activity of the enzyme was stimulated up to 6-fold within few hours, when *Medicago* roots were preincubated with Nod factors at nanomolar concentrations. Roots preincubated with *R. meliloti* showed similar stimulation of the hydrolase. Mutant strains, unable to produce Nod factors, did not stimulate the enzyme activity (Staehelin et al., 1995). The hydrolase had a Michaelis-Menten constant for Nod factors in the micromolar concentration range. Chitotriose or chitotetraose, added to the assay mixture at 500-fold higher concentration than the Nod factor substrates, did not show any inhibitory effect on Nod factor degradation. When chitotetraose was tested as substrate for the enzyme preparation, only a low degradation activity was observed indicating that the acyl chain of Nod factors enhances the hydrolytic reaction. The enzyme was unable to release detectable amounts of chitooligosaccharides from colloidal chitin. These data show that we characterized a new plant enzyme specific for the cleavage of lipo-chitooligosaccharides.

Staehelin et al. (1995) Plant Physiol. *in press*

INDUCTION OF THE SUBTERRANEAN CLOVER CHALCONE ISOMERASE GENE FAMILY BY *RHIZOBIUM* INFECTION

WEINMANN, J.J., SHOOBRIDGE, M.V., ARIOLI, T., AND ROLFE, B.G.
Center for Genetic Research (Plant Microbe Interaction Group),
Research School of Biological Sciences,
Australian National University, A.C.T. 0200, Australia.

In *Trifolium subterraneum,* chalcone isomerase (CHI) is encoded by a multi-gene family with three members. We have designed and used a set of degenerate PCR primers to amplify three separate fragments from within genomic *CHI* genes. These fragments differ in size due to a variable sized intron between the priming sites, and can be distinguished by the presence of specific restriction sites within this intron. These PCR fragments have been used to probe a subterranean clover genomic library (Arioli et al. 1994). It was found that the three *CHI* copies, designated Ts*CHI-A*, Ts*CHI-B* and Ts*CHI-C,* are contained on two overlapping lambda clones and are clustered within a 15 kb region. These genes were subcloned, mapped and we are now completing their molecular characterization by sequencing. The Ts*CHI-B* and *-C* copies are similar but have much less homology to the *A* copy. In turn, the Ts*CHI-A* copy has greater homology to the bean *CHI* gene (Blyden et al. 1991) than to the *-B* or *-C* copy. The structure of the Ts*CHI-A* copy varies from the bean *CHI* gene in that in subclover the first intron is absent.

We have previously shown that chalcone synthase in subterranean clover is induced by *Rhizobium* in a copy specific manner (Lawson et al. 1994). We have used a 3'-RACE procedure to demonstrate that *Rhizobium* infection also induces CHI. When cDNA made from subterraneanclover roots 6 h after *Rhizobium leguminosarum* bv *trifolii* strain ANU843 inoculation is used as template in a PCR reaction primed with the upstream degenerate primer and a 3'- anchor primer, three fragments are produced. In contrast, cDNA made from a control inoculation produces no amplification products. A ~800 nt fragment has been confirmed as a partial *CHI* transcript by hybridization. Restriction analysis of this fragment indicates that it is likely to be produced from either the Ts*CHI-B* or *-C* gene copy.

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NODULATION FACTORS DIFFER IN THEIR POTENTIAL TO PROMOTE MYCORRHIZAL COLONIZATION OF SOYBEANS

XIE, Z.-P., STAEHELIN, C.[1], VIER-HEILIG, H.[2], WIEMKEN, A.,
JABBOURI, S., BROUGHTON, W.J., VOGELI-LANG, R.,
AND BOLLER, T.
Botanisches Institut der Universität Basel, Hebelstrasse 1, CH-4056 Basel, Switzerland (Z.-P.X., C.S., H.V., A.W., R.V.-L., T.B.); and L.B.M.P.S., Université de Genève, 1 chemin de l'impératrice, CH-1292 Chambésy/Genève, Switzerland (S.J., W.J.B.)

[1] Present address: Institut des Sciences Végétales, CNRS, Avenue de la Terrasse, F-91198 Gif-sur-Yvette Cedex, France

[2] Present address: Faculté de Foresterie et de Géomatique, Université Laval, Pavillon C. E. Marchand, Quebec G1K 7P4, Canada

We investigated synergistic effects between rhizobia and the vesicular-arbuscular mycorrhizal fungus *Glomus mossae* in the rhizosphere of soybean. Mycorrhizal colonization of several soybean cultivars was considerably enhanced when the plants were co-inoculated with ***Bradyrhizobium japonicum*** or ***Rhizobium sp.*** NGR234. Ineffective ***Bradyrhizobium*** strains induced a similar stimulatory effect on mycorrhiza indicating that a functional rhizobial symbiosis is not necessary for the observed increase of fungal colonization. However, inoculation with the mutant strain ***Rhizobium*** sp. NGR*ΔnodABC* ,which is deficient in the production of Nod factors,did not promote mycorrhizal colonization of soybeans. When a preparation containing a mixture of partly purified Nod factors from NGR234 was added to the watering solution, the degree of mycorrhizal colonization was enhanced whereas a corresponding preparation from NGR*ΔnodABC* did not show any effect on mycorrhiza formation. Highly purified Nod factors from ***Rhizobium sp.*** NGR234 differed in their potential to promote fungal colonization. The acetylated Nod factor NodNGR-V(MeFuc,Ac), added at concentrations as low as 10^{-9} M, was active whereas the sulfated Nod factor NodNGR-V (MeFuc, S) was inactive. Preliminary results indicate that Nod factors did not stimulate the germination of isolated spores from *Glomus mossae*. However, several soybean flavonoids known to be accumulated in the rhizosphere in response to NodNGR-V(MeFuc,Ac) induced a similar promoting effect on mycorrhizal colonization of soybeans. These results suggest that the synergistic effect between rhizobia and mycorrhiza is mediated by host plant flavonoids. Interestingly, a non-nodulating soybean mutant blocked in the root hair deformation response showed also a Nod factor induced stimulation of mycorrhizal colonization. Moreover, various soybean cultivars with a low symbiotic potential, characterized by the formation of less than 3 nodules per plant, showed a similar enhancement of mycorrhiza formation by Nod factors. These data suggest that all tested soybeans were able to perceive Nod factors and to respond to them with an increased susceptibility to vesicular-arbuscular mycorrhiza.

BIOCHEMICAL RESPONSES OF SOYBEAN CELLS INDUCED BY NOD FACTORS FROM *BRADYRHIZOBIUM JAPONICUM*

YOKOYAMA, T.(1), KOBAYASHI, N.(2), KOUCHI, H.(1), MINAMISAWA, K.(2), KAKU, H.(1)
(1) National Institute of Agrobiological Resources, Kannondai 2-1-2, Tsukuba, Ibaraki, 305, Japan
(2) Ibaraki University, Amimachi 3998, Inashiki, Ibaraki, 300-03, Japan

The Nod factors evoke various responses of the plants within a few hours or a day at pico- to nanomolar concentration. And furthermore, for example, concerning *R.meliloti* Nod factors, the presence of a sulfate group at the reducing end of the sugar backbone is essential for activity on the host plant *Medicago*. These facts suggest that the Nod factors could act through a receptor on the leguminous plant cell membrane. However, the receptor and signaling pathways of the Nod factors are still unknown.

In order to clarify the biochemical responses of soybean cells to Nod factors, changes in cytosolic Ca^{2+} level of four kinds of plant cells after addition of Nod factors were measured by using a fluorescent Ca^{2+} indicator of Fura-PE3.

Soybean root cell (*Glycine max* cv. Enrei) and three kinds of microcallus cells (*Glycine max* cv. Enrei, *Nicotiana tabacum* cv. Blight Yellow BY-2, and *Oryza sativa* cv. Kinmaze) were incubated with 2μmol of Fura-PE3 AM (a membrane-permeant ester derivative of Fura-PE3) in order to load those cells with Fura-PE3. The Nod factor of *B.japonicum* TARC64 was purified and used to confirm the biochemical responses of soybean cells to the Nod factor. Nod Bj-V ($C_{18:1}$,Me-Fuc) and NodBj-V ($C_{19:1}$) of USDA 110 were kindly given by Dr.G.Stacey of the Tennessee University and used to confirm the structural reguirements for their biological activity.

When the Nod factor was applied to the two kinds of cells of *Glycine max* cv. Enrei, those two types of cells showed sharp spikes of fluorescence emission caused by the increase of cytosolic Ca^{2+} concentration within 2 minutes. While, BY-2 and Kinmaze cells did not show any increase of fluorescence emission on the addition of the Nod factor. These results suggest that early Nod factor-mediated signals are transduced through cytosolic calcium, and Nod factor of *B.japonicum* are specifically working to the soybean cells. To confirm the structural reguirements for biological activity of Nod factors, Nod Bj-V ($C_{18:1}$,Me-Fuc) and NodBj-V ($C_{19:1}$) of USDA 110 were applied to soybean micro callus cells, respectively the cells treated with Nod Bj-V ($C_{18:1}$,Me-Fuc) showed definite increase of fluorescence emission at 2 min after treatment. Whereas, the cells treated with NodBj-V ($C_{19:1}$) did not show any significant change. This means the presence of a methyl-fucosyl group at the reducing end of the sugar backbone is essential for biological activity of Nod factors.

OUTER CORTICAL SWELLING IN SOYBEAN ROOTS CAUSED BY *BRADYRHIZOBIUM ELKANII*

YUHASHI, K (1), AKAO, S (2), FUKUHARA, H (1),
CHUN, J(3), STACEY, G(3), HARA, H(1), KUBOTA, M(1)
and MINAMISAWA, K(1,3,4)
(1)School of Agricul.,Ibaraki Univ.,Ami,Ibaraki,300-03 Japan.(2)Nat. Inst. of Agrobiol. Resources,Tsukuba,Ibaraki,305 Japan.(3)Center for Legume Res.,Dep.Microbiol.,UT, Knoxville,TN 37996-0845 USA. (4)Inst. of Genetic Ecology, Tohoku Univ., Sendai, Miyagi,980 Japan.

When *Glycine max* cv. Enrei roots were inoculated with *B. elkanii* USDA76, a localized swelling of root subepidermal cortical cells was observed within 10 days after inoculation. The swelling was formed from outwardly enlarged plant cells, which were 60 to 300 µm in length and were located immediately adjacent to the epidermis. Because the root swelling was caused by an enlargement of the outer cortical cells, the swelling was termed outer cortical swelling (OCS). The OCS often extended from the outermost layer of the cortex to two or three cell layers inside. Inoculation of soybean roots with *B. elkanii* USDA94 or USDA31 also induced OCS. In contrast, no OCS was observed in roots inoculated with *B. japonicum* USDA110 and USDA122 and in uninoculated controls. Therefore, the OCS response appears to be unique to roots inoculated exclusively with *B. elkanii*. To determine the involvement of the nodulation genes in OCS formation, we constructed a Nod$^-$ mutant, strain USDA94_NOD, of *B. elkanii* USDA94 in which the nodD_2D_1KABC genes were deleted. An OCS response was observed on roots inoculated with USDA94_NOD, indicating that the lipo-chitin nodulation signals synthesized by the *nodABC* gene products are not required for OCS formation. The fact that USDA94_NOD was capable of eliciting an OCS response prompted us to examine the involvement of *B. elkanii* IAA production. Previous research (Minamisawa, Fukai, 1991) indicated that IAA excretion is an exclusive property of free-living *B. elkanii* and is not found in *B. japonicum*. For this experiment, we used strain TN3, an IAA-deficient mutant derived from *B. elkanii* USDA31 after chemical mutagenesis (Fukuhara et al.,1994). Soybean roots inoculated with *B. elkanii* strain TN3 did not induce OCS, while roots inoculated with its parent strain USDA31 exhibited a clear OCS response under the same conditions. Although the mutation in strain TN3 has not been genetically characterized (Fukuhara et al.,1994), the result suggests that IAA and its related compounds produced by *B. elkanii* may be involved in the elicitation of the OCS response on soybean roots.

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IDENTIFICATION OF EARLY NODULATION SIGNALS IN *LOTUS JAPONICUS*

ZHANG, Z.[1], SCOTT, B.[2], STACEY, G.[3], GRESSHOFF, P.M.[3]
[1]Laboratories of Agro- Microbiology, Huazhong Agricultural University, Wuhan, 430070 China. [2]Dept. of Microbiology and Genetics, Massey University, Palmerston North, New Zealand. [3]Center for Legume Research, The University of Tennessee, Knoxville, TN37901-1071, USA.

Lotus. japonicus can be used as a model plant for structune -function analysis in nodulation and nitrogen fixation. *Rhizobium loti* is a fast-grower, which nodulates many *lotus* species. We used *R. loti nod*A-*lac*Z fusion to monitor early nodulation signals in *Lotus japonicus*. Expression of the *nod*ABC operon was measured by assaying β-galactosidase activity in strain PN1372(NZP2037 containing *nod*A-*lac*Z fusion). The results showed that *R. loti nod*A-*lac*Z fusion was able to be induced by seed exudates of *Lotus japonicus*. The studies of Kinetics of β-galactosidase activity began to rise after inducing for 60 minutes, reaching a peak at 6 hours and then slightly reduced; the β-galactosidase activity increased with seed exudates concentration up to 1/6. 25 dilution. This results indicated that the inducer existed in the seed exudates of *L. japonicus*.

Analysis of extracts of nodule, root, stem, leaf, seed coat and cotyledon of *L. japonicus* showed signal levels characteristics for each tissue type. The inducing activity produced by root extract was highest. The seed coat extract was second in order. The activities of extracts of nodule, stem and cotyledon were low. The activity of leaf extract approached to naught. To determine which of the flavonoids was able to induce *R. loti* nodulation gene operon, We tested for response to 17 different flavonid compounds. But all of them showed no inducing activity. HPLC fractions from *L. japonicus* seed exudates were tested on the *nod*A-*lac*Z fusion. The inducing activity appeared in two succeding fractions eluting 5-8 minutes in 25-30% methanol. The spectrum range was 190nm. The structure of inducer will be analyzed further.

PHOSPHORUS NUTRITION ALTERS ROOT FLAVONOID CONTENT, NITROGEN FIXATION AND PHOSPHORUS PARTITIONING IN COWPEA

DAKORA F.D. AND LE ROUX R.J.
Dept. of Botany, University of Cape Town, Rondebosch 7700, South Africa

Root nodule formation in legumes begins with the release of plant flavonoids that induce transcription of nodulation (*nod*) genes in *Rhizobium* and *Bradyrhizobium* bacteria. Most of the studies done so far on the synthesis, release and/or quality of nod gene-inducing compounds from nodulating legumes have used palnt culture systems that are nutrient-sufficient. By contrast, agricultural soils differ in the levels of nutrient elements available to plants. Typically, phosphorus is deficient in many soils and limits legume plant growth and nodule functioning. Studies with some species have shown that increasing P supply to symbiosis-dependent legumes such as soybean (*Glycine max* L.), pea (*Pisum sativum* L.), and stylosanthes (*Stylosanthes humilis* H.B.K.) increases various indices of N_2 fixation, suggesting more specific effects of P on nodule formation and functioning than on plant growth processes. But it is not clear from those studies whether providing P to the legumes promoted the formation of nod gene-inducing flavonoids, decreased the synthesis of molecules that inhibit nod gene induction, and/or enhanced a spacific symbiotic process in bacteroids which resulted in increased N_2 fixation. This study examines the effects of P nutrition on root flavonoids, symbiotic performance, and P partitioning in cowpea. Cowpea seedlings, inoculated with *Bradyrhizobium* strain CB 756, were cultured hydroponically using N-free nutrient solution contaaining two levels of P, O and 2 mM. The nodulated crown zone of each plant was always maintained above the nutrient solution. At 34 DAP, plants were harvested for assessing nodulation and N_2 fixation, and for determination of P levels in shoots, whole nodules, nodule cortex and the bacteria-infected tissue. Total flavonoid levels were measured spectroscopically at an absorbance of 250 nm, using 80% MeOH extracts of cowpea roots from the different P treatments. The amount of root flavonoids (expressed in daidzein equivalence, daidzein being a major flavonoid in cowpea and an active nod gene inducer in its microsymbiont increased significantly (p=0.05) in 2 mM-grown cowpea plants, and coincided with significant (p=0.05) growth, nodulation and N_2 fixation in these plants compared to those at 0 mM P, suggesting that the improvement in P nutrition probably resulted in a rise in the production and/or quality of nod gene-inducing flavonoids that enhanced nodulation. Tissue P concentrations were also about 32-fold greater in shoots of plants grown in 2 mM P compared to 0 mM P. The amount of P allocated to each single nodule from 2 mM P treatment was 16.8 mg compared to 0.8 mg at 0 mM P. At 2 mM, where P supply was sufficient for normal plant growth and development of optimal symbiosis, the concentration of P in bacteroid tissue was about twice that in the nodule cortex.

PREINCUBATION OF RHIZOBIUM INOCULANTS WITH FLAVONOIDS ENHANCES NODULATION IN CHICKPEA AND PEANUT

HOOPER, W., SWAMINATHAN, R., PALANINAPPAN SP.,
THOMAS, J.
Centre for Biotechnolgoy, SPIC Science Foundation 110 Mount Road,
Guindy, Madras - 600 032, India

Flavonoids, from legume roots are the initial signal molecules in the chemical communication system in the legume - *Rhizobium* symbiosis. Such signal molecules have not been identified in chickpea, a major legume crop in India. We have isolated nodulation inducers from chickpea seeds and obtained enhancement in nodulation by exposing the inoculant rhizobia to host seed exudate or specific nodulation inducer flavonoids.

As *nod-lacZ* construct is not available in chickpea rhizobia, we have used the following bioassay. *Rhizobium* IC76 was grown in the presence of different fractions of seed exudate; the seedlings were inoculated with the cell-free culture filtrates and assessed for root hair deformation/curling activity . To isolate nodulation inducer compounds, seed exudate was first extracted with diethyl ether. The ether fraction was separated by TLC and the a major spot showing good Had/Hac activity was fractionated by HPLC using a C_{18} reverse phase column with a methanol-water gradient and the active peaks identified. Alternatively, ethanol extract of the seed powder was fractionated by HPLC. One of the active peaks comigrated with biochanin A in methanol-water gradient. This compound also comigrated with biochanin A in acetonitrile-water-acetic acid gradient indicating that this compound may be biochanin A. Biochanin A, daidzein, genistein and isoliquiritigenin also induced *nod* factors in chickpea rhizobium.

We have examined the concept that pretreatment of inoculant rhizobia with seed preparation or appropriate nodulation inducer flavonoids, may enhance nodulation in legumes. Addition of seed exudate to Rhizobium ciceri IC76 or F75 either in the growth medium or at the time of injecting into vermiculite packets or at the time of coating the seeds with inoculant, enhanced the number of nodules formed significantly in chickpea. Addition of specific flavonoids also enhanced nodulation significantly. Rhizobia pre-exposed to seed preparations / flavonoids in the vermiculite carrier were used as inoculant in peanut and soybean field trials. Plants inoculated with rhizobia specifically preconditioned with host seed preparation or specific nodulation inducer flavonoid often significantly enhanced nodulation, shoot biomass and grain yield.

DEGRADATION AND MODIFICATION OF *NOD* GENE-INDUCING FLAVONOIDS BY RHIZOBIA.

RAO J.R., COOPER J.E.
Department of Applied Plant Science, The Queen's University of Belfast,
Newforge Lane, Belfast BT9 5PX, Northern Ireland.

A number of *Rhizobium* species and biovars have been shown to degrade their *nod* gene-inducing flavonoids by mechanisms which originate in a cleavage of the C-ring of the molecule. The initial products of these reactions are new open and (re)closed ring flavonoid structures. Subsequent C-ring fissions yield a variety of conserved A- and B-ring monocyclic aromatics (Rao, Cooper, 1994). Further studies concentrating on the degradation of the isoflavone *nod* gene inducers daidzein and genistein by *Bradyrhizobium japonicum* have identified a wider range of metabolites which can be classified into three groups: C-ring modification, conserved A-ring and conserved B-ring products. The first group included isoliquiritigenin, liquiritigenin and coumestrol from daidzein and naringenin chalcone, naringenin and coumestrol from genistein. Conserved A-ring products from daidzein were resorcinol and umbelliferone and from genistein phloroglucinol, phloroglucinol carboxylic acid and 4-methyl umbelliferone. Conserved B-ring products included 4-hydroxybenzoic acid, 4-hydroxycinnamic acid and phenylacetic acid. All products were detected in extracted supernatants of inducer-supplemented (10μM) *B. japonicum* cultures after incubation periods ranging from 2 to 24 h. Liquiritigenin, naringenin, coumestrol and umbelliferone were also found in *B. japonicum* cells. Root exudates incubated with *B. japonicum*, *R. fredii* or *Rhizobium* sp. NGR234 exhibited substantial quantitative changes in their isoflavonoid profiles. These results raise the possibility of a role for some modification/degradation products in *nod* gene regulation and in the recognition mechanisms operating in the early phases of communication between the symbiotic partners.

Rao JR, Cooper JE (1994) J. Bacteriol. 176, 5409-5413.

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BIOCHEMICAL ANALYSES OF FLAVONOIDS IN ROOTS AND ROOT EXUDATES OF STERILE AND INOCULATED *LOTUS PEDUNCULATUS* SEEDLINGS.

STEELE H.J. (1), BJOURSON A.J. (1), RAO J.R. (1), WETZEL A. (2), WERNER D. (2), COOPER J.E. (1).
(1) Department of Applied Plant Science, The Queen's University of Belfast, Newforge Lane, Belfast BT9 5PX, Northern Ireland. (2) Fachbereich Biologie, Philipps-Universität Marburg, Karl von Frisch-Strasse, 35032 Marburg, Germany.

Flavonoids are the primary molecular signals from the host legume to free-living rhizobia. A reciprocal influence of rhizobia on flavonoid biosynthesis in legumes has been proposed to explain qualitative and quantitative changes in the root exudate flavonoids of inoculated plants (Recourt et al., 1991; Dakora et al., 1993). To examine the effect of *R. loti* on the flavonoid content of roots and root exudates of *L. pedunculatus* in the early phase of symbiotic interaction we have employed a wide range of analytical techniques including TLC, HPLC, capillary electrophoresis (CE) and GC-MS. Together with TLC, CE proved to be particularly suitable for separating flavonoids in methanolic extracts of root tissues and a spectral array detector provided absorption spectra of separated peaks over a wavelength range of 195-365 nm. Inoculated roots were extracted and analysed five days after adding rhizobia and sterile roots of the same age were treated in the same way. Results indicated no significant qualitative differences in the flavonoid content of the two types of root. Root exudate analyses detected up to eight flavonoid structures under sterile conditions, with quercetin prominent among them. Preliminary data from exudates of inoculated plants indicate that flavonoid content may display qualitative differences compared with sterile samples, which could be explained by Nod factor-mediated release of new compounds into the rhizosphere (Schmidt et al., 1994) and/or rhizobial modification/degradation of existing flavonoids (Rao, Cooper, 1994).

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THE LPS-PLASMIDS FROM *R. etli*, *R. leguminosarum* bv. *trifolii* AND *R. leguminosarum* bv. *viceae* ARE FUNCTIONALLY AND STRUCTURALLY RELATED

GARCIA DE LOS SANTOS, A., GIRARD, M.L. AND BROM, S.
Department of Molecular Genetics, Centro de Investigacion sobre Fijacion de Nitrogeno, UNAM. Ap.Postal 565-A, Cuernavaca, Mor., Mexico.

Molecular genetics research in *Rhizobium* has focused mainly on the study of genetic information carried on the symbiotic plasmid, specifically in those genes that participate in the symbiotic process (*nod, nif* and *fix* genes). Outside this symbiotic region, our knowledge is very limited. What is the global role of the large quantity of plasmid-encoding genetic information? As an approach to the study of the structural and functional relationship among *Rhizobium* plasmids, in our laboratory we are characterizing the *R. etli* CFN42 plasmids. This strain, which forms nodules on roots of common bean, contains six plasmids (pa to pf) ranging in size from 150 to 600 kb. One of these plasmids, different from pSym, called p*R.etli* CFN42b (180kb), encodes functions related to lipopolysaccharide (lps) biosynthesis, indispensable for nodule formation. We have shown that this genetic region is highly conserved structurally as well as functionally only in plasmids of *R. etli, R. leguminosarum* bv. *trifolii* and *R. leguminosarum* bv. *viceae* strains, and absent in other *Rhizobium* and *Agrobacterium* species. Looking for a structural relationship among these three plasmids, we have done plasmid- incompatibility studies, which show that p*R.etli* CFN42b and p*R.leg.* bv. *trifolii* ANU843b belong to the same incompatibility group, suggesting a close- ancestr al origin. Southern-blot hybridization experiments with total DNA from ANU843 (wild and derivative cured of plasmid b) and VF39 (wild and derivative cured of plasmid c) strains, using the p*R.etli* CFN42b or its cosmid clones as probes, show that these three plasmids contain a large number of common sequences. The above data show that in addition to the *lps* locus, genetic information from p*R.etli* CFN42b is highly conserved in the genome of *R. leguminosarum*. Although *R.etli* and *R.leguminosarum* belong to taxonomically diverse lineage, with different geographical origin and host-range, they share extrachromosomal sequences, suggesting that they may be equally- advantageous in the presence of comparable selective pressures.

DELETION OF A *BRADYRHIZOBIUM JAPONICUM* EPS GENE CLUSTER IMPAIRS THE SYMBIOTIC INTERACTION WITH HOST PLANTS

KOSCH, K., BATINIC, T., *NIEHAUS, K., WERNER, D., AND MULLER, P.
Philipps-Universität, FB Biologie, Karl-von-Frisch-Str., 35032 Marburg, Germany
*Universität Bielefeld, FK Biologie, Postfach 100131, 33501 Bielefeld, Germany

The sequence of a genomic region of *Bradyrhizobium japonicum* 110*spc*4 encompassing four open reading frames (ORF1 to ORF4) was determined. All ORFs are transcribed in the same direction. The deduced amino acid sequences share significant homology with gene products involved in the exopolysaccharide (EPS) biosynthesis of other microorganisms. The gene product of ORF1 and ORF2 are homologous to ExoP of *Rhizobium meliloti* (Becker *et al.*, 1993) However, the *exoP* gene of *B. japonicum* 110*spc*4 consists of two seperate ORFs as has also been shown for homologous genes of other micororganisms. ORF3 putatively codes for a glycosyltransferase because this function has been suggested for the homologous gene in *Streptococcus pneumoniae* (Guidolin *et al.*, 1994) ORF4 encodes for a UDP-1,4-galactose epimerase and shows the highest degree of homology to the respective genes of other organisms (Buendia *et al.*, 1991).

A deletion in the *B. japonicum* mutant strain ΔP22 affecting all of these ORFs leads to an altered composition and structure of the exopolysaccharide. A high resolution chromatography of wild-type and mutant EPS revealed that the high molecular weight fractions are hardly detectable, whereas low molecular weight material is overproduced compared in the mutant strain. The LPS of the mutant strain remains unaffected (Parniske *et al.*, 1993). After the inoculation with *B. japonicum* 110*spc*4 a disturbance of the nodulation process on various host plants can be detected. This altered phenotype is dependent on the genotype of the host plant and is due to plant defense reactions (Parniske *et al.*, 1994, Kosch *et al.*, 1994).

The *exoP*1and *exoP*2 mutant strains *B. japonicum* ES12 and EH3 were generated via vector integration mutagenesis. The disturbance of the nodulation process of *Glycine max* cv. Maple Arrow inoculated with these strains is even more pronounced than with *B. japonicum* ΔP22, where the deletion affects the extreme C-terminal part of *exoP*1 and *exoP*2. At 21 dpi tiny nodules have developed mainly at the site of lateral root growth. Most of them have a white or green central nodule tissue. These nodules do not fix nitrogen as shown by the acetylene reduction assay, whereas *B. japonicum* ΔP22 induced nodules are as effective as wild-type nodules. These results further confirm the importance of the EPS structure for the nodulation process of determinate nodule type plants.

TRANSCRIPTIONAL ORGANIZATION AND EXPRESSION OF HOST-SPECIFICITY GENES FROM *RHIZOBIUM FREDII.*

KOVACS L.G., BALATTI P.A., KRISHNAN H.B., PUEPPKE S.G.
Department of Plant Pathology, University of Missouri, Columbia, MO 65211 USA

Rhizobium fredii fixes nitrogen in association with soybean and a number of other legume species. We have studied the transcriptional organization of a sym plasmid locus that functions negatively to restrict nodulation of some soybean cultivars by *R.fredii* strain USDA257. Inactivation of any of the genes of this host-specificity locus, *nolXWBTUV*, allows mutants to form nitrogen fixing nodules on the soybean cultivar McCall.

nolX and *nolW* are oriented right-to-left, and the remaining genes are oriented left-to-right. The initiation codon for *nolW* is 155 bp upstream from that of *nolB*; *nolX* lies downstream of nolW and is separated from it by 281 bp. Primer extension analysis confirms that the genes of the *nolXWBTUV* locus are transcribed from three promoters. Two of these, which are between *nolW* and nolBTUV, face one another and initiate transcription at sites that are only 14 bp apart. The third lies directly upstream from *nolX*. The *nolW* promoter is constitutive in cultured bacteria, whereas the *nolB* and *nolX* promoters are inducible by flavonoid signal compounds.

We have conducted experiments to express genes from this locus in *Escherichia coli* systems. We detected the insert- and orientation-specific expression of two genes, *nolX* and *nolW*, with the T7 RNA polymerase/promoter system, but we were unable to express *nolBTUV*. Similar results were obtained in an vitro system with *E.coli* S-30 extract. We raised antiserum against NolT- and NolX-fusion proteins and used them to detect abundantly expressed polypeptides of the predicted sizes of the NolT and NolX proteins in protein extracts of USDA257. These polypeptides were only present in flavonoid-induced cells. These observations, as well as RNA dot blot data from a series of mutamts, indicates that *nolBTUV* is flavonoid-inducible and expressed as a single transcriptional unit in *R.fredii*. Flavonoid-inducibility is one of the most peculiar aspects of the expression of genes in the *nolXWBTUV* locus. *nolX* and *nolBTUV* have this propert, which is dependent on the regulatory gene *nodD*, yet neither is preceded by a *nod* box promoter region of the type normally thought to be crucial for *nodD*-dependent flavonoid-induction. Moreover, the *nolX* and *nolBTUV* promoter regions do not share conserved sequences, and thus their flavonoid-inducibility must be mediated by different promoters. We are currently subjecting the regions upstream of both of these genes to deletion analysis, in an effort to pinpoint sequences necessary for responsiveness to flavonoids.

GENETIC CHARACTERIZATION OF A *Rhizobium meliloti* CHROMOSOMAL DNA REGION INVOLVED BOTH IN LIPOPOLYSACCHARIDE (LPS) BIOSYNTHESIS AND IN SYMBIOSIS.

LAGARES, A.[1], NIEHAUS, K.[2], LORENZEN, J.[2], HOZBOR, D.[1],
PICH OTERO, A.J.L.[1] AND PUHLER, A.[2]

(1) Instituto de Bioquímica y Biología Molecular (IBBM), Facultad de Ciencias Exactas, Universidad Nacional de La Plata, calles 47 y 115, 1900-La Plata, Argentina.

(2) Lehrstuhl für Genetik, Fakultat für Biologie, Universität Bielefeld, Bielefeld, Germany.

There are a number of reports which indicate that lipopolysaccharides (LPS) are necessary for root infection and nodule development in different rhizobia-legume associations. LPS mutants of *Rhizobium leguminosarum* bv vicie, *R. leguminosarum* bv trifolii, *R. leguminosarum* bv phaseoli and *Bradyrhizobium japonicum* have been described to be altered in their plant infection ability. Our studies with *R. meliloti* indicated that in this rhizobia LPS deficiencies also can lead to impaired symbiotic phenotypes. A Tn*5* LPS mutant derived from *Rm*2011 - Rm6963 - showed a decreased competitiveness for nodulation of alfalfa (Lagares et al., 1992, J. Bacteriol. **174**:5941-5952) and a Fix$^-$ phenotype when inoculated on *Medicago truncatula* (Niehaus et al., in preparation). On these two host plants indeterminate nodules are formed. To advance in our studies on the symbiotic role of *R. meliloti* LPS, we initiated the genetic analysis of *Rm*6963. Molecular cloning of a Tn*5* containing DNA fragment from the mutant indicated that the transposon insertion maps in the chromosome within the previously described *lpsB* gene. Reverse mutagenesis and complementation experiments showed that the Tn*5* insertion in *lpsB* is responsible for both the LPS alteration and the symbiotic changes in this rhizobia. Southern-blot hybridization using as probe a cloned DNA fragment containing the *lpsB* gene, revealed a conserved 5.5 kbp *Sst*I DNA fragment in all tested *R. meliloti* from different geographic origins. Sequence analysis of this fragment showed that in the upstream region of *lpsB* there is an open reading frame homologous (59%) to the *Escherichia coli* and *Rickettzia prowatzekii* transcription factor gene *greA*. We have not investigated if this gene is also in *Rhizobium* related to antitermination activity. Insertional mutagenesis and vector integration analysis indicated that *greA* and *lpsB* would be part of two different transcriptional units. In agreement with the experimental evidences, a sequence homologous to the "non-nitrogen" promoter consensus [TTP$_u$ANN 16-17b P$_u$A(P$_u$)$_4$ 3-5b CA] was found between *greA* and *lpsB*. Expression studies by using chromosomal *lpsB-lacZ* transcriptional fusions showed that the *lpsB* gene is expressed in free living conditions with variations along the phase growth of culture. Sequencing of the 3 kbp DNA region downstream *lpsB* showed the presence of four putative ORFs, some of them possibly related to LPS biosynthesis. We are currently investigating the symbiotic phenotype of bacterial mutants carrying alterations in each of these genes.

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GENETIC ANALYSIS OF A CLUSTER OF GENES REQUIRED FOR EXOPOLYSACCHARIDE SYNTHESIS IN *RHIZOBIUM ASTRAGALUS*

SONG, H-Y, HUANG, J.-B, SHU, W.-Y., AND HUA, Z.
Shanghai Institute of Plant Physiology, Academia Sinica, Shanghai 200032, CHINA

Acidic exopolysaccharide (EPS) of rhizobia has been proved essential for establishing a successful invasion and nodule development on legumes which develop indeterminate nodules (Gray *et al.*, 1990). We have previously identified *Rhizobium astragalus* from nodules of indeterminate type on its host, *Astragalus sinicus* L. (Chen *et al.*, 1990). We also reported an extensive set of Tn5 insertion mutants of *R. astragalus* that fail to produce normal amount of Calcofluor-binding, acidic EPS. The mutants manifest different symbiotic deficiency (Chen *et al.*, 1991; Long et al., 1992).

In this paper we describe the construction of R-prime plasmids carrying genetic segments responsible for EPS production of *R. astragalus*. One of such plasmid (exoR'-11) derived from Exo mutant, NA-11, was able to complement group A mutants. All the Exo$^+$ transconjugants formed Fix$^+$ nodules as did the wild-type strain. ExoR'-01 and exoR'-11 were characterized in more detail: the Tn5 insertions were ascertained, the relative positions of the sub-fragments within the entire fragment of two exoR's(38.2kb and 36.5kb) were mapped. The defective Ndv$^-$ phenotype of group A mutants could be corrected by 2.7kb PstI-BamHI fragment from exoR'-11. Complementation analysis revealed the presence of at least five loci being involved in *R.astragalus* EPS synthesis. Mutations affecting production of EPS were clustered on the genome of megaplasmid 2 in *R. astragalus*.

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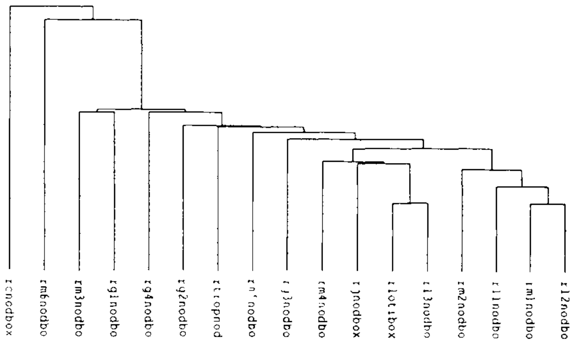
IDENTIFICATION OF NODULATION GENE CLUSTERS IN *RHIZOBIUM GALEGAE* STRAIN HAMBI 1174

SUOMINEN, L.[1], JURGENS, G.[1], ROOS, C.[2], PAULIN, L.[2],
AND LINDSTROM, K.[1]
[1]Department of Applied Chemistry and Microbiology, P.O.Box 27, FIN-00014 University of Helsinki, [2]Institute of Biotechnology, P.O.Box 45a, FIN-00014, University of Helsinki, Finland

Galega sp. is a temperate legume which is nodulated only by *Rhizobium galegae*, a narrow host range rhizobial species described by Lindström (1989). The nodulation genes of *Rhizobium galegae* strain HAMBI 1174 have been cloned and identified in two separate clusters. The cosmid pRg30, sized 26 kb, contains common nodulation genes *nod*ABCIJ and a regulatory *nod*D1 gene, whereas pRg10, a 28 bp cosmid clone, carries a second *nod*D homolog and three of the four *nod*-boxes present in this strain. *R. galegae* belongs to the same phylogenetic branch with agrobacteria (see Lindström *et al.*, this volume). According to the sequence data and the protein alignments obtained from the *nod* gene region, the common nodulation genes *nod*A, B and C of *R. galegae* are closest related the corresponding genes of *R. leguminosarum* biovars *viciae* and *trifolii* and of *Sinorhizobium meliloti*.

A PileUp comparison shows the position of the four *R.galegae nod*-boxes within rhizobial *nod*-box sequences from different sources. Further analysis will reveal the relevance of this data in connection with rhizobial evolution.



PileUp sequence comparison of various nod-boxes found upstream from the nod operons.

ANALYSIS OF GENES INVOLVED IN EPS I BIOSYNTHESIS OF *RHIZOBIUM MELILOTI*: *EXOR*/*EXOP** MUTANTS PRODUCING NO DETECTABLE HIGH MOLECULAR WEIGHT EPS I ARE ABLE TO INDUCE EFFECTIVE NODULES ON ALFALFA

BECKER A., NIEHAUS K., KÜSTER H., PÜHLER A.
Universität Bielefeld, Fakultät für Biologie, Lehrstuhl für Genetik, Postfach 100131, D-33501 Bielefeld, Germany

Rhizobium meliloti 2011 produces the acidic exopolysaccharide succinoglycan (EPS I), which is essential for the infection of alfalfa root nodules. A high molecular weight (HMW) and a low molecular weight (LMW) form of EPS I is produced by *R. meliloti.* 19 *exo* genes involved in EPS I biosynthesis are clustered in a 24 kb region on megaplasmid 2. The combination of genetic and biochemical approaches resulted in a detailed model of the biosynthesis of the EPS I subunit (Leigh, Walker, 1994). In contrast, much less is known about polymerization and export of EPS I. To date the membrane associated proteins ExoP, ExoQ and ExoT have been identified to be involved in these stages of EPS I biosynthesis (Leigh, Walker, 1994).

The *exs*A gene encoding a protein homologous to ABC transporter proteins was identified adjacent to the *exo*B gene. *R. meliloti* strains mutated in *exs*A were characterized by a decreased ratio of HMW to LMW EPS I indicating a function of ExsA in EPS I biosynthesis (Becker et al., 1995b).

The membrane topology of the ExoP protein was analysed by translational fusions of *lac*Z and *pho*A reporter genes to the *exo*P gene (Becker et al. 1995a). Based on these experiments, the ExoP protein could be divided into an N-terminal domain mainly located in the periplasmic space and a C-terminal domain located in the cytoplasm. Whereas the C-terminal domain of ExoP is characterized by a potential ATP/GTP binding motif, the N-terminal ExoP domain contains a sequence motif of 32 amino acids ($RX_4P/AX_2PX_4SPKX_{11}GXMXG$), which was also present in proteins involved in the determination of O-antigen chain length (Becker et al., 1995a). *R. meliloti* strains characterized by mutated *exo*P* genes exclusively encoding the N-terminal ExoP domain produced a reduced amount of succinoglycan. This reduction could be suppressed by a mutation in the regulatory gene *exo*R (Becker et al., 1995a). The proportion of low molecular weight to high molecular weight succinoglycan was significantly increased in the *exo*P* mutant strains, which leads us to suggest that ExoP might be involved in the determination of the ratio of high molecular weight to low molecular weight EPS I. The *exo*R/*exo*P* mutant produced large amounts of LMW EPS I, whereas no HMW EPS I could be detected in the culture supernant of this mutant. The *exo*R/*exo*P* mutant was able to invade alfalfa nodules (Becker et al., 1995a).

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Becker A. et al. (1995b) submitted for publication
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LPS MUTANTS OF *Rhizobium fredii*

BUENDIA, A.M. (1), VILLALOBO, E. (1), KOPLIN, R. (2), TORRES, A. (1), PUEPPKE, S.G. (3), BREWIN, N.J. (4) AND RUIZ SAINZ, J.E. (1)
(1) Dep. Microbiologia, Fac. Biologia, Univ. Sevilla. Apdo 1095 41080 Sevilla, Spain. (2) Dep. Genetik, Univ. Bielefeld, Germany. (3) Dep. Plant Pathology, Univ. Missouri, USA. (4) Dep. Genetics, John Innes Centre, Norwich, UK.

Considerable evidence exists indicating that the cell surface polysaccharides of *Rhizobium/Bradyrhizobium* species are essential for the establishment of a functional symbiosis. Our model organism is *Rhizobium fredii*, a fast-growing nitrogen- fixing symbiont of wild and cultivated species of the genus *Glycine*. In addition, *R. fredii* nodulates other species of legumes. In order to study the role of *R. fredii* LPS in symbiosis, we have isolated several LPS mutants and analysed the symbiotic phenotypes conferred by these mutations. We have constructed monoclonal antibodies that recognize the LPS of *R. fredii* strain HH103. Monoclonal antibody NB3/206.57 recognizes the typical ladder pattern on the LPS I region and two bands in the LPS II region. LPS mutants of *R. fredii* strain HH103 have been isolated by transposon mutagenesis and screening of colonies showing an altered morphology. Mutant strain L105 has a Tn5 insertion in a 11Kb *Eco*RI fragment and it is not recognized by NB3/206.57. The ladder of the LPS I shows a higher electrophoretic mobility and the LPS II region has only one band with a mobility which is in between the two LPS II bands of the wild type strain. Mutant strain L178 has a Tn5-Mob insertion in a 14Kb *Eco*RI fragment, it is still recognized by the antibody, the LPS I bands have a higher electrophoretic mobility and it has lost the upper band of the LPS II region. Mutant strain L105 formed nodules on *Glycine max*, *Macroptilium atropurpureum* and *Cajanus cajan* but there was a clear reduction of the number of nodules and nitrogen fixation. This mutant formed ineffective nodules on *Desmodium canadense* and *Indigofera tinctoria* and failed to nodulate *Macrotiloma axillare*. Mutant L178 formed pseudonodules on *G. max*, *M. atropurpureum* and *D. canadense* and failed to nodulate *C. cajan*, *M. axillare* and *I. tinctoria*. These results provide further support for the hypothesis that an intact LPS is essential for the development of determinate nodules.

INFLUENCE OF EXOPOLYSACCHARIDES ON LUTEOLIN UPTAKE BY *RHIZOBIUM MELILOTI*

C. HUBAC (1), J. FERRAN (1) and A. KONDOROSI (1),(2).
(1) Institut des Sciences Végétales, CNRS,91198, Gif/Yvette Cedex, France.
(2) Institut of Genetics, Biological Research Center, Hungarian Academy of Sciences, 6701 Szeged, Hungary.

The characteristics of luteolin uptake by *R. meliloti* was studied in mutants affected in exopolysaccharide and Kps production and in the wild type strain. In the mutants affected in exopolysaccharide production, the luteolin uptake was strongly enhanced at acidic pH (Hubac et al., 1994a). Introduction of a plasmid containing *exoB* genes into one of these *exoB* strains resulted in a strain overproducing the exopolysaccharides which exhibited practically the same characteristics for luteolin uptake as the wild type strain. Consequently, exopolysaccharides play a role in luteolin uptake, probably by constituting an efficient barrier against an excess of flavonoids. The analysis of bacterial growth in the presence of luteolin at different concentrations confirmed this hypothesis: the *exoB* strains were susceptible to a weak luteolin concentration. The presence of Kps did not affect either luteolin uptake or bacterial growth. To prove that the exopolysaccharides are involved in this process, the high molecular weight exopolysaccharide fraction from *R. meliloti* was added to the culture of an *exoB* mutant strain. In these conditions, a marked decrease of luteolin uptake was recorded in comparison with the culture of the *exoB* mutant strain. Moreover, comparative biochemical analysis of outer membranes in wild type and mutant strains showed that the *exoB* mutation strongly affected the lipopolysaccharide composition: in absence of *exoB* genes coding for the glucose-epimerase, LPS backbone contains glucose instead of galactose (Williams at al. 1990). We think that the lack of EPS induces modifications in LPS pattern. However in the strain where the EPS were restored (bright with the calcofluor test), the LPS pattern was always similar to the strain *exoB*.

In conclusion:

- the higher luteolin uptake at acidic pH in *exoB* strain,
- the bacterial growth with increasing concentrations of luteolin,
- the restoration of EPS in a strain *exoB*,
- the addition of exogenous EPS in a culture of *exoB*

confirm the hypothesis (Hubac et al., 1994b) that EPS play a protective role against an excess of luteolin.

Hubac C. et al., (1994a) 1st European Nitrogen Fixation Conference, Szeged, 310; G.B. Kiss, G. Endre G. ed.
Hubac C. et al., (1994b) Microbiology, **140** 2769-2774
Williams et al., (1990) Journal of bacteriology, **172**,2622-2632

LUTEOLIN UPTAKE BY *RHIZOBIUM MELILOTI* MUTANTS AFFECTED IN LYPOPOLISACCHARIDES PRODUCTION AND NODULATION COMPETITIVENESS.

HUBAC, C.[1], FERRAN, J.[1], SHARYPOVA L.A.[2]. AND ONISHCHUK, O.P.[2]
(1) Institut des Sciences VÂgÂtales, C.N.R.S., 91198, Gif/Yvette Cedex, France ; Fax (33 1) 69 82 3695.
(2) All-Russia Research Institute for Agricultural Microbiology, Podbelsky Shaussee 3, St Petersburg-Puskin 6, 189620 Russia; Fax + 7 812 470 4362.

Flavonoids uptake is a good trait to study the early steps of the establishment of symbiotic interaction between *R. meliloti* and *Medicago*. Especially luteolin uptake by mutants affected in LPS production and nodulation competitiveness was studied. Luteolin uptake was always pH dependent; it was higher at acidic pH than at neutral or basic pH. Great differences were found among mutants.

- 1) The LPS I- mutants (Zatovska et al., 1994) showed weak luteolin uptake at acidic pH, in agreement with the hypothesis that LPS could be a good candidate to absorb luteolin in its non-ionized state at acidic pH in the lipidic matrix of the membrane (Hubac et al., 1994a).

- 2) Other mutants affected in nodulation competitiveness, which were LPS^+ and EPS^+ (Onishchuk et al., 1994), absorbed very low amount of luteolin, independent of the pH.

These results are discussed in relation with EPS production and the ability to nitrogen fixation: in the first case, an Exo^- strain with altered LPS showed a luteolin uptake higher than Exo^+ LPS^- strain, but lower than Exo^+ LPS^+ strain. The protective role of EPS against an excess of flavonoid is confirmed (Hubac et al., 1994b). Moreover, mutants affected in LPS production are often impaired in their competitiveness, but retain Fix^+ Eff^+ phenotype . Howere, there is a relation between competitiveness and luteolin uptake, even in the case when LPS are uneffected.

Zatovska TV et al. (1994) Ist European Nitrogen Fixation Conference, Szeged, 47; G.B. Kiss, G. Endre ed.
Hubac C. et al. (1994a) Microbiology, 140, 2769- 2774
Hubac C. et al. (1994b) Ist European Nitrogen Fixation Conference, Szeged, 310; G.B. Kiss, G. Endre, ed.
Onishchuk O.P. et al. (1994) Plant and Soil 167 267-274

INTERCONNECTION OF SOME BIOCHEMICAL AND FUNCTIONAL PROPERTIES OF LIPOPOLYSACCHARIDES FROM *RHIZOBIUM LEGUMINOSARUM* BV. *VICIAE*

KOSENKO L.V., ZATOVSKA T.V.
Department of Biochemistry of Microorganisms, Institute of Microbiology and Virology, National Academy of Sciences of Ukraine, Kiev, 252143, Ukraine

Lipopolysaccharides(LPS) isolated by phenol-water extraction from 10 strains of *R.leguminosarum* bv. *viciae* (symbionts of pea and broad bean plants) were purified from low molecular heteroglycans by gel filtration on Sepharose 4B (LPS-1) followed by ultracentrifugation(105000 x g) to release from glucan (LPS-II). LPS, LPS-I and LPS-II were subjected to mild hydrolysis with 1% acetic acid. Lipopolysaccharides were shown to be separated from glucan more easily in effective than in non-effective strains at two variants of procedure (ultracentrifugation and mild specific hydrolysis with 1% acetic acid). The analysis of the ratio and monosaccharide composition of fractions obtained by gel filtration of partially degraded polysaccharides (PS) of LPS has revealed some peculiarities of lipopolysaccharide complex depending on symbiotic properties of *R. leguminosarum* bv. *viciae*. O-chaincontaining LPS (S-form) dominated over R-LPS, and fractions of free O-polysaccharide (O-PS) or its large molecular fragments were present in acetic acid hydrolysates of LPS from the most highly effective strains (252b, 97, 95). The R-LPS prevailed in the most highly virulent bacteria(strains 248b and 87). The LPS of effective strains more actively interacted with host plant lectins compared with lipopolysaccharides from non-effective rhizobia. Some suppositions were done on the basis of these data. Labile links of both glucans with LPS or O-chain with core (or within O-PS itself) may have the functional significance. Separation of glucan from LPS after penetration of rhizobia through cell wall of root hairs possibly opens LPS for their interaction with plant lectin. Free O-polysaccharide fragments of LPS may play role of signal molecules. Domination of S-LPS in lipopolysaccharide complex of *R.leguminosarum* bv. *viciae* guarantees the presence of signal molecules binding lectin of gomological host plant.

LPS CHANGES IN *R. MELILOTI* EFB1 AS A RESPONSE TO IONIC AND OSMOTIC STRESS.

LLORET, J.[1], BOLANOS, L.[1], LUCAS, M.M.[2], PEART, J.M.[2],
BREWIN, N.J.[2], BONILLA, I.[1], AND RIVILLA, R.[1]

1. Dept. Biología. Universidad Autónoma de Madrid. 28049 Madrid. Spain. 2. John Innes Center. Norwich NR4 7UH. U.K.

Bacterial adaptation to salty environments is acomplished by the intracellular accumulation of compatible solutes to increase the internal osmotic pressure and avoid cytoplasmic dehydration. Among rhizobia *R. meliloti* is one of the most halotolerant and the accumulation of several osmotica has been reported. Although these mechanisms can explain the osmotic tolerance, the toxic effects of ions must be overcome by other means. We have focused on the role of lipopolysaccharide (LPS) in the adaptation of *R. meliloti* to salt, since adaptation of rhizobia to different environmental and physiological situations is related to structural changes in the LPS.

We have isolated a *R. meliloti* strain (EFB1) from nodules of a *Melilotus sp.* plant growing in a salt marsh in Doñana National Park. EFB1 is able to grow at NaCl concentrations up to 500 mM and no effect on growth is produced by 300 mM. When grown on solid medium supplemented with NaCl the morphology of the colonies changed resembling the semi-rough colonies produced by LPS mutants, indicating the possibility of salt-inducible alterations on the LPS structure. Futhermore, NaCl concentrations that did not affect groth (300 mM) induced alterations on the LPS electrophoretic profile which were evident after silver staining. The alterations consisted on different mobilities of the LPS1 fraction and the appearance of new bands on the O-antigen. The same alterations were induced by other salts but not by polyethylene glycole (PEG 200)a non-ionic, non-metabolizable osmoticum. However, a monoclonal antibody raised against EFB1 cells grown with and without salt supplementation, recognized a sugar epitope on cells grown in the presence of different salts and PEG 200. This epitope was not present on cells grown on non-supplemented medium.The intensity of the immunostaining was proportional to the salt concentration and was maximun on cells grown with PEG 200, indicating that this epitope was osmotically induced.

These LPS alterations should be complementary to the accumulation of compatible solutes and should be regarded as an adaptative mechanism to saline environments.

ON THE INVOLVEMENT OF LIPOPOLYSACCHARIDES IN BACTERIA-PLANT CONTACT INTERACTIONS FOR NITROGEN FIXING ASSOCIATIVE BACTERIA OF THE GENUS *AZOSPIRILLUM*

MATORA L.YU., SOLOVOVA G.K., SEREBRENNIKOVA O.B., SELIVANOV N.YU., AND SHCHYOGOLEV S.YU.
Laboratory of Physical Chemistry of Cell Structures, Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, Saratov 410015, Russia

The establishment of associative interrelationships between free-living microorganisms and plants does not result in the formation in plants of any clearly detected morphological structures (like nodules, tumors, etc). It follows that while evaluating the involvement of *Azospirillum* lipopolysaccharides (LPS) in bacteria-plant contact interactions, one is forced to choose some informative physiological and biochemical tests to characterize the results of such interactions. We used experimental data on: i) adhesion of azospirilla to wheat roots and carrot cells in suspensions; and ii) induction of plant responses to bacterial inoculation which were revealed by the peculiarities of the synthesis and enzyme activity of specific polypeptides in the wheat root apoplast. The effect of pretreatment of plant material with isolated *Azospirillum* LPS on some characteristics obtained with taking account of the data listed was investigated. Such a pretreatment enhanced carrot cell agglutination as affected by bacterial cultures and increased ten-fold the number of the cells attached to wheat roots. Consequently, as distinct from the known experiments on agrobacteria, the pretreatment did not inhibit the adhesion process of the azospirilla. Moreover, even the pretreatment itself led to a marked increase in the synthesis of major polypeptides in the wheat root apoplast. The effect appeared to be comparable with the analogous plant response to inoculation with intact cells. We also recorded enhanced chitinase and cellulase activities in response to both plant inoculation with bacterial cultures and plant treatment with the preparations of the isolated LPS. The activities of pectinase and β 1,3-gluconase did not change with certainty. Note, that the pretreatment was performed with considerable excess of LPS which presumably leads to its fairly high concentration on the surfaces of plant cells and tissues. Because of this, enhanced bacterial adhesion both to carrot cells and to wheat roots may be explained, for example, by the effect of electrostatic interactions between acidic fractions of O-specific polysaccharides and the basic proteins that are components of the outer bacterial membrane. This assumption was verified by experiments on self-agglutination of cells of *A. brasilense* Sp 245 under the effect of LPS isolated from different *Azospirillum* strains (this strain including). It is apparent that during the relatively short time period typical of these experiments (about several tens of minutes for agglutination or several hours for bacterial adhesion), the so-called “non-specific” bonds between the components of plant and microbial cells are most likely to manifest themselves. They do not require specific partner responses like the adduced changes in the composition of plant metabolites. Thus, it may be concluded that *Azospirillum* LPS are actively involved in associative plant-bacteria interactions both on the level of non-specific bacterial adhesion and on the level of induction of specific plant responses.

WHEAT LECTIN REGULATES NITROGEN METABOLISM IN *AZOSPIRILLUM BRASILENSE*.

ANTONYUK, L., FOMINA, O. AND IGNATOV, V.
Institute of Biochemistry & Physiology of Plants & Microorganisms, Russian Academy of Sciences, Entuziastov av. 13, Saratov, 410015, Russia

One of the wheat metabolites, viz. lectin (wheat germ agglutinin, WGA) is stimulatory to nitrogen fixation in *Azospirillum brasilense* Sp 245, a natural wheat colonist (Antonyuk et al., 1993). The lectin appeared to increase ammonia excretion and glutamine synthetase (GS) activity in the bacterium and to depress the inhibitory effect of O_2 on the nitrogenase as well. The dependencies of N_2-fixation, ammonia excretion and GS activity on lectin concentration had a similar pattern and correlated well with each other (the correlation coefficients ranged from +0.83 to +0.98). We proposed that the binding of wheat lectin to the cell surface receptors of *Azospirillum* was followed by transmembrane transduction of a signal to the regulatory systems that are responsible to derepression of biosynthesis of glutamine synthetase and nitrogenase. To test this suggestion, we prepared extracts of WGA-incubated and non-incubated *A.brasilense* cells grown under N_2-fixing conditions. Both suspensions were equal in volume and cell number prior to incubation and were thoroughly washed off after incubation. The absence of the lectin admixure was tested by the hemagglutination reaction. Rocket immunoelectrophoresis of the extracts with antibodies against GS of *A. brasilense* Sp 245 demonstrated that wheat lectin caused the biosynthesis of the enzyme and not its activation. The above extracts were also analysed by SDS-PAGE and it was shown that the protein profile of WGA-incubated cells had 64 bands against 26 bands in the control. The densitometry of protein profiles displayed that in the case of WGA-incubated cells, Mo-Fe protein of nitrogenase had a peak area of 32 mm^2 against 12 mm^2 in the control, and this, in its turn, attests to the lectin-induced biosynthesis of nitrogenase. These findings invoke in favour of the expression of rather a large number of genes as a bacterial response to WGA. Since WGA stimulates main processes that determine the efficiency of the wheat-*Azospirillum* association, N_2-fixation with following excretion its product and indole acetic acid production (Iosipenko et al, 1994), the lectin possibly serves as a signal molecule to support the effectiveness of the association.(Supported by the Russian Foundation for Basic Research, Grant No. 95-04-11473).

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WHEAT GERM AGGLUTININ RECEPTOR OF *AZOSPIRILLUM LIPOFERUM:* SEPARATION AND FUNCTION

KARPATI, E.[1], KISS, P.[2], AFSHARIAN, M.[1], DEL GALLO, M.[3] AND FENDRIK, I.[4]

Departments of [1]Biotechnology and [2]Chemistry and Biochemistry, Gödöllõ University of Agricultural Sciences, H-2103 Gödöllõ, Hungary; [3]DISTAAM, Faculty of Agriculture, University of Molise, I-86100 Campobasso, Italy; [4]Institute of Biophysics, University of Hannover, D-30419 Hannover, Germany

Lectin wheat germ agglutinin (WGA) is found on wheat embryos and root tips of adult wheat plants. In the colonization of wheat roots by *Azospirillum*, WGA can represent a particular binding site for the receptors of bacteria. In this attachment bacterial capsule components and organella, such as proteins/glycoproteins, polysaccharides, fimbriae and flagella are supposed to be involved (Del Gallo, Fendrik, 1994). Moreover, nitrogenase and glutamine synthetase activities and ammonia excretion have also been shown to be triggered by binding of WGA in *A. brasilense* (Antonyuk et al., 1993).

Recently a 25 kDa putative WGA-receptor protein/glycoprotein has been detected in the capsule of *A. lipoferum*. WGA-nonbinding (WGA$^-$) mutant has also been isolated and the receptor encoding DNA region has been localized (Karpati et al., in press).

In the present work this WGA-receptor protein was isolated by affinity chromatography. The Triton-X solubilized capsule of *A. lipoferum* was passed over WGA - Sepharose CL-4B column. Components adsorbed were eluted and examined by SDS-polyacrylamide gel electrophoresis. It showed a 25 kDa adsorbed glycoprotein consistent in molecular mass with the previously detected putative WGA-receptor (Karpati et al., in press).

Stimulation of nitrogenase activity both with WGA and Sambucus nigra agglutinin revealed that N-acetylglucosamine type of binding to the receptor is involved in the modification of the enzyme activity.

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ASSOCIATIVE NITROGEN-FIXING BACTERIUM *AZOSPIRILLUM BRASILENSE* 75: ADSORPTION TO WHEAT (CV. SARATOVSKAYA 29) ROOTS AND BACTERIAL POLYSACCHARIDE-CONTAINING COMPONENTS THAT PRESUMABLY AFFECT *AZOSPIRILLUM* -WHEAT ROOT INTERACTIONS

KONNOVA, S.A., ROGOVA, T.A., PROKHOROVAR, N.,
EGORENKOVA, I.V., GUSHCINA, S.G., MAKAROV, O.E.,
SKVORTSOV, I.M. AND IGNATOV, V.V.

Institute of Biochemistry and Physiology of Plants and Microorganisms,
Russian Academy of Sciences, Pr. Entuziastov 13, Saratov 410015, Russia

The local associative nitrogen-fixing bacterial strain *Azospirillum brasilense* 75, isolated from wheat (*Triticum aestivum* cv.Saratovskaya 29, Russia) roots (Pozdnyakova et al 1988), was shown to display marked adsorption to the root surface using *in vitro* experiments on sterile seedlings of this cultivar. With the time of contact with the bacterial suspension ranging from 15 min to 48 h, the number of adsorbed cells, that reached the end of the logarithmic phase of growth, increased progressively up to the 24 h exposure and then remained at the attained level (ca.$4x10^6$ cells/cm root). The adsorption curve was similar to that described by Michiels et al (1991) for anchoring of *A.brasilense* Sp7 to wheat (*Triticum aestivum* cv.Fidel (SES, Belgium) roots. The bacteria *A.brasilense* 75 induced root hair deformation in 2-day-old seedlings of the wheat Saratovskaya 29. To elucidate the possible role in this process of extracellular high-molecular polysaccharide-containing substances, we have isolated a lipopolysaccharide-protein complex (LPPC) and polysaccharide-lipid complex (PSLC) as previously described (Konnova et al ,1994). By using a slide technique, it was shown that the LPPC and PSLC over the concentration range between 0.1 and 0.4 mg/ml. caused changes in root hair morphology. We explored the ability of complexes to inhibit wheat germ agglutinin (WGA) which is known to be present in the roots of wheat seedlings. The LPPC and PSLC at concentrations of 5.1 and 7.7 mg/ml, respectively, were found to inhibit WGA by 50%. An acidic polysaccharide (PS), isolated from the PSLC, at a concentration of 0.06 mM (3 mg/ml) caused 50% inhibition of WGA activity. The PS was composed of rhamnose (72%), galactose (24%), xylose (3%), galacturonic acid (3%), glucosamine(1%), and galactosamine (4%). Comparison between the activities of LPPC and PSLC, isolated from other strains, with respect to the ability to induce root hair deformation has led us to assume that the bacteria display strain selectivity for the host plant. We speculate that at least one of the steps in the *Azospirillum*-wheat root interaction may be determined by WGA affinity for the PS of the extracellular PSLC.

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EFFECT OF ORGANIC SUBSTANCES EXUDED FROM ROOTS OF NON-LEGUMES ON PLANT CONTROL OF ASSOCIATED NITROGEN FIXING BACTERIA.

KRAVCHENKO, L.V., AZAROVA, T.S., MAKAROVA, N.M.
AND LENOVA, E.I.
All-Russia Research Institute for Agricultural Microbiology
Podbelsky Shosse 3, St. Petersburg, 189620
Russia

The constitution and the activities of the microbial communities in rhizosphere are determined to a great extend by the chemical composition of their environment. The goals of the present study are to 1) determine the effect of plant genotype on the root colonization process based on the energy demand of nitrogen fixing bacteria; 2) study the effect of root exudates on the ability of associated diazotrophs to optimize the plant mineral nutrition; 3) determine the role of volatile seed and root exudates as a factor impacting on selection of rhizosphere microorganisms; 4) determine the efficiency of hormone biosynthesis by associated diazotrophs during the interaction between bacteria and plants. We have shown that the total number of diazotrophs with respect to the amount of carbon in the root exudates of various plant genotypes is 20-50 times higher for diploid (wild type) varieties of wheat as compared with the hexaploid (selected type) ones. This is correlated with the increased content of organic acids and low concentration of amino acids and carbohydrates in root exudates. Volatile exudates caused dramatic increase in growth of diazotrophs, however they inhibited their nitrogenase activity by a factor of 40-70. Hormone biosynthesis in the rhizosphere is considerably determined by the presence in root exudates of some suitable compounds which may be the precursors of auxin and cytokinins synthesis. In all this cases a careful selection of bacteria-plant pairs for effective response to inoculation of plant with associated diazotrophs is required. The same approach is necessary for active colonization of plant roots by introduced strains. On the basis of our results we elaborated the principles for selecting the pairs of the bacteria inocula and the host plants for creating optimal conditions for plant growth.

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GENETIC ANALYSIS OF AN *AZOSPIRILLUM BRASILENSE* SP7 MUTANT IMPAIRED IN FLOCCULATION.

PEREG, L.L.[1,2,] MILLET, J.[1], KATUPITIYA, S.[2], KENNEDY, I.R.,[2] AND ELMERICH, C.[1]
(1) Unité de Pysiologie Cellulaire, Département des Biotechnologies, URA 1300 CNRS, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France.
(2) Department of Agricultural Chemistry and Soil Science, University of Sydney, Sydney 2006 Australia.

Azospirillum brasilense can fix nitrogen in association with agriculturally important crops. A spontaneous mutant of *A. brasilense* Sp7, Sp7-S, was found to reduce acetylene at higher rates than the wild type in association with wheat roots. The mutant strain formed smaller colonies, did not bind congo red, did not flocculate, lacked capsular polysaccharides, did not form cysts and stayed in the vegetative state when associated with roots (Katupitiya et al., 1995).

To characterize the genetic defect of Sp7-S a gene bank of DNA fragments of strain Sp7 was introduced by conjugation into Sp7-S and a plasmid clone, pAB1220, which restored congo red-binding and flocculation to Sp7-S was isolated. The complementing region was subsequently localised by Tn*5* mutagenesis. Two Tn*5* insertions, situated at 0.4 kb from each other, in a 1.1 kb *Pst* I fragment abolished the complementaton, and the 1.1 kb fragment was sufficient to restore congo red binding ability and flocculation.

The two Tn*5* insertions were subsequently recombined in the host genome of Sp7. The resulting mutant strains were unable to bind congo red and to flocculate. Colonization of wheat roots was studied by inoculation of wild type and Tn*5* induced non-flocculating mutants containing a constitutive *lacZ* fusion. It was found that both Tn*5* mutants were impaired in colonization of wheat roots and the colonization ability was fully restored by complementation with the 1.1 kb fragment.

Katupitiya et al. (1995) Appl. Environ. Microbiol. 61, 1987-1995.

ISOLATION AND CHARACTERIZATION OF A CHVE HOMOLOGOUS PROTEIN IN *Azospirillum brasilense* Sp7

VAN BASTELAERE, E., KEIJERS, V., AND VANDERLEYDEN, J.
F.A. Janssens Laboartory of Genetics, Willem de Croylaan 42,
B-3001 Heverlee, Belgium

To study the role of the host plant in the associative interaction between *Azospirillum* and the plant, protein profiles of bacteria grown in the absence and in the presence of plant exudates were compared on 2D-PAGE.

Several proteins seemed to be induced in *Azospirillum* when the bacteria were grown in the presence of the plant. Most obvious was the induction of a 40 kDa acidic protein (Van Bastelaere et al., 1993).

Nucleotide sequence analysis of the corresponding gene of *A. brasilense* Sp7 has been performed. At the amino acid level the protein showed 74% homology with the ChvE protein of *A. tumefaciens* (Huang et al., 1990).

A promotor::*gusA* fusion has been constructed and introduced in *A. brasilense*. The *gusA* gene is expressed in the presence of wheat root exudates and of different sugars, inducing the *A. tumefaciens chvE*.

A *chvE* mutant has also been constructed in *A. brasilense*. Mutant analysis will be performed and will help us to elucidate the role of the ChvE protein in the interaction between Azospirillum and the plant.

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PHYSIOLOGICAL AND BIOCHEMICAL MECHANISMS OF THE FORMATION OF LEGUME- *RHIZOBIUM* SYMBIOSIS

ANDREJUK, K.I. AND ANTIPCHUK, A.F.
Inst. Microbiol. and Virol., Ukraine Acad. of Sci., Zabolotny 154, Kiev, 252143, Ukraine

The first stages of legume-*Rhizobium* symbiotic relations include the recognition of partners. During this period, the formation of symbiosis depends upon lectin-carbohydrate interaction, fermentative activity of rhizobia, including pectinolytic activity, and rhizobial resistance to antibiotics that are presented in the seeds of legume plants. Lectin- carbohydrate interaction of the partners has been studied. In particular, it was shown with *R. leguminosarum* that EPS of rhizobia can induce nodule formation. The degree of affinity of rhizobial LPS's to the host plant lectins was higher for the effective strains and it was connected with the existence of functionally important sugars in the O-chain composition of these polymers.

Rhizobial efficiency correlated significantly with the pectinase activity. Thus, when in contact with host-plant seeds, total pectinolytic activity of the highly efficient strains of *R. leguminosarum*, *R.sp.*(*Lupinum*) and *Br. japonicum* was 6-25 times higher than in cultures. When in contact with sprouts of plants of responsive varieties, it is 20-78% higher than that observed in contact with sprouts of irresponsive varieties. Positive correlation between rhizobial pectinolytic and nodulation activities was determined. Probably, pectinase activity may be considered as one of the inducers of the rhizobial efficiency.

Exudates of legume plant seeds also take part in symbiosis formation. It has been determined that seed coat extracts of pea, lupin and soybean irresponsive varieties could inhibit rhizobial growth. Inhibiting substances are thermostable; they can be extracted with various dissolvents, and are phenol-containing. We assume that adaptive selection of rhizobial strains is one of the ways for their efficiency improvement.

SECTION 5

SYMBIOTICALLY ESSENTIAL BACTERIAL GENES AND DYNAMICS OF *RHIZOBIUM* GENOME

WHAT IS STILL TO BE LEARNED ABOUT THE *RHIZOBIUM* GENOME AND *RHIZOBIUM* GENES INVOLVED IN SYMBIOSIS ?

HYNES* M.F., MITSCH M.J., FAAS L.A., AND YOST C.K.
Dept. of Biological Sciences, University of Calgary, Calgary, AB,
Canada, T2N 1N4

Although tremendous progress has been made in understanding nodulation of legumes and nitrogen fixation in legume nodules at a molecular level, there are still many unanswered questions in this field of research. The presentations in the following session are very diverse in their content, and deal with some of the important unanswered questions dealing with the biology of the rhizobia. Some of the questions which will be addressed in this plenary session, and which are the subject of active investigation in many laboratories, include: 1) Why is the *Rhizobium* genome arranged in the way it is, and what is the role of all the various components of this genome ?
2) Why is there so much reiteration of the *Rhizobium* genome ? 3) Are there as yet undiscovered genes directly involved in nodulation or nitrogen fixation, whose presence has thus far been masked by the fact that these genes are reiterated? 4) What are the mechanisms by which differentiation into bacteroids occurs, and what are the pathways that control this differentiation ?

In our laboratory, we are attempting to address several of these questions. We have been attempting to find out the role of various plasmids in *R.leguminosarum* by using an approach which selects directly for plasmid curing (Hynes et al. 1989). We have shown that in some strains, three different plasmids are absolutely required for a functional symbiosis (Hynes, McGregor, 1990) and ascribed numerous other functions, including a large number of loci involved in sugar catabolism, to various plasmids (Baldani et al. 1992, Faas, Hynes, unpublished). Similar work carried out in other labs (Charles, Finan, 1991, Brom et al. 1992) has given similar results. What we do not yet know is why there are so many large plasmids in *Rhizobium* spp., and whether there is any significance to the fact that certain genes, including many involved in symbiosis, are plasmid-encoded. *Bradyrhizobium* spp.(which have all their symbiotically important genes on their chromosomes) function equally well in a similar ecological role so there appears to be no *a priori* reason why these genes should be extrachromosomal in *Rhizobium* . In an attempt to try and understand this, our lab is studying both the variability and ecological importance of plasmid-encoded catabolic genes.

One consequence of the complexity of the *Rhizobium* genome seems to be that there is a large amount of reiteration of genes. This raises the possibility that , since the primary method which has been used to identify symbiotically important genes has been transposon mutagenesis, many duplicated genes have been missed. Recent work on *nodD* ,*nodPQ* , *nodM,* and other genes has confirmed this as a possibility. In our lab, work on *fix* genes has shown that there are two functional copies of both the

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fixHGIS and the *fixNOQP* operons. These copies are located on two different plasmids. It thus appears highly likely that several novel symbiotically important genes remain to be discovered. The approaches which may work to find these genes include differential screening/ subtraction hybridisation based on expression of genes in response to eg. flavonoids, deletion of large segments of plasmids or chromosomes, and plasmid curing.

One area where a great deal of work remains to be done is the area of differentiation into bacteroids. It has been shown that a number of different changes take place in LPS structure (Satyavir et al. 1990), outer membrane proteins (de Maagd et al. 1994), and of course in expression of *nif* and *fix* genes once a cell has differentiated into a bacteroid, but we know little about the signalling events which give rise to these changes. It has been conclusively shown that oxygen plays a key role in regulation nitrogen fixation (see paper by Batut et al, this volume), but it seems inherently unlikely that oxygen concentration is the only signal involved in differentiation into bacteroids. For example, bacteroids are apparently devoid of flagella, so presumably flagellar synthesis is switched off. However, if low oxygen concentration were the signal that causes this down-regulation, free-living cells under hypoxic conditions would lose their flagella and thus their ability to escape from the unfavourable hypoxic conditions. This is not logical from an evolutionary point of view.

In our lab, we are currently searching for possible signals which could switch off certain cellular processes during symbiosis. The system we have chosen to work with is the chemoreceptors (Methyl-accepting chemotaxis proteins: MCPs), which are unlikely to be required in bacteroids. The genes coding for these proteins are thus likely to be down regulated during symbiosis. much like flagellar genes. We are also interested in the ecological role these chemoreceptors might play. We have identified a family of at least 7 MCP genes by PCR and hybridisation, and cloned five of these. Sequencing has revealed a high degree of homology to *Caulobacter* MCPs , with signalling domains, methylation sites, and transmembrane domains all being highly conserved. Mutations in two of these putative MCPs have been made, and have been shown to affect chemotactic behaviour. It is now our intention to create gene fusions to MCP genes, and to study their regulation during symbiosis. It is our hope that these fusions can be used as probes to identify signals other than oxygen concentration which affect gene expression during development of bacteroids. This is obviously only one strategy for looking for new signalling pathways. Others, as mentioned, could involve outer membrane proteins or flagellar synthesis genes.

A combination of the methods outlined above (large deletions, differential screening, and use of appropriate gene fusions to detect down regulation of specific processes during symbiosis) appears to us to be likely to yield a wealth of new information about what additional genes are important to symbiotic life of rhizobia, and some useful new insights into the regulatory events which induce rhizobial cells to differentiate into bacteroids.

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DYNAMICS OF THE *Rhizobium* GENOME.

PALACIOS R., CASTILLO, M., FLORES M., HERNANDEZ G.,
MAVINGUI P. AND ROMERO D.
Department of Molecular Genetics, Centro de Investigación
sobre Fijación de Nitrógeno. Universidad Nacional Autónoma
de México,
Ap. postal 565-A, Cuernavaca, Mor. México.

INTRODUCTION

The *Rhizobium* genome can be considered as a complex structure, formed by the chromosome and high molecular weight plasmids, that contains a large amount of reiterated DNA sequences and presents frequent rearrangements. A general approach to detect reiterated DNA sequences estimated in *Rhizobium etli* the presence of 700 reiterated elements belonging to 200 different families; high levels of DNA reiteration were also observed in *R. meliloti* and in *Agrobacterium tumefaciens* (Flores et al, 1987). This degree of DNA reiteration is unusual among bacteria; only in *Archaebacteria* and in *Streptomyces* such large amounts of reiterated DNA sequences have been shown (Hopwood, Kieser, 1990; Krawiec, Riley, 1990). Independent of its specific biological significance, reiterated DNA sequences function as sites for homologous recombination resulting in genomic rearrangements.

The occurrence of frequent deletions as a consequence of recombination between insertion elements in a symbiotic region of the chromosome of *Bradyrhizobium japonicum* has been reported (Hahn, Hennecke, 1987). Flores et al (1988) and Brom et al (1991) followed direct approaches to detect frequent genomic rearrangements in *R. etli.* Individual subclones derived from a single cell were analyzed in regard to their hybridization patterns against different probes or by their plasmid profiles. Differences were found in about 3% of the subclones. Amplifications, deletions, loss of plasmids and cointegration of plasmids were detected.

The recent construction of genetic elements (GDYN elements) that allow the positive selection of different types of rearrangements (Romero et al, 1991) has greatly improved the analysis of these phenomena. Using such

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elements we have found that gene amplification is a frequent event in different regions of the genome in *R. etli* (Romero et al, 1991 and 1995; Flores et al, 1993) and of *R. leguminosarum* bv. *trifolii* (Stepkowski et al, unpublished results). This prominent capacity for the generation of gene amplification is the crucial feature in our proposition of a dynamic model of the *Rhizobium* genome.

DYNAMIC MODEL OF THE *Rhizobium* GENOME.

The basic units of this model (Fig. 1) are regions of the genome that have the potentiality to be amplified. Such units or amplicons are bordered by repeated DNA sequences present in direct orientation. Homologous recombination between these sequences results in either a duplication or a deletion of the whole amplicon region. The frequency of duplication varies for different amplicons and may be related to the lenght and the degree of homology of the direct repeated sequences bordering the amplicon.

The rate limiting step for amplification is the initial duplication event. Once the duplication step is achieved, the structure formed is a tandem direct repeat of the whole amplicon region. Such structure may recombine leading to amplification or to deletion of one of the units. Due to the fact that duplicated or amplified regions are tandem arrays of perfect homology, they behave as very reactive structures in regard to recombination, continuously increasing or decreasing the amplification level and eventually returning to the non amplified state. During this dynamic process the structure of the genome is not disrupted and the DNA sequence in conserved. Moreover, in amplicons that duplicate and amplify at very high frequency, the DNA sequence of the direct repeats bordering the amplicon will show a tendency to be maintained highly homologous or even identical.

It is important to point out that as a result of its high recombination activity amplified regions are continuously liberating from the genome closed circular structures that contain monomers or multimers of the whole amplicon sequence. Since these structures usually do not contain an origin of replication they are diluted out during the growth of the strain. However, these circular units may also participate in further recombination events leading to other genomic rearrangements.

The first amplicon described in *Rhizobium* corresponds to a nod-nif region of the symbiotic plasmid of *R. etli* (Romero et al, 1991). This amplicon has a lenght of 120 kb and presents as borders two 5kb direct repeated sequences that correspond to the two functional nitrogenase operons previously reported for this *Rhizobium* species (Quinto et al, 1985; Romero et al 1988). This amplicon duplicates at a frecuency of about 10^{-3} (Romero et al, 1991) and a direct analysis of subclones of strain CFN42 has shown

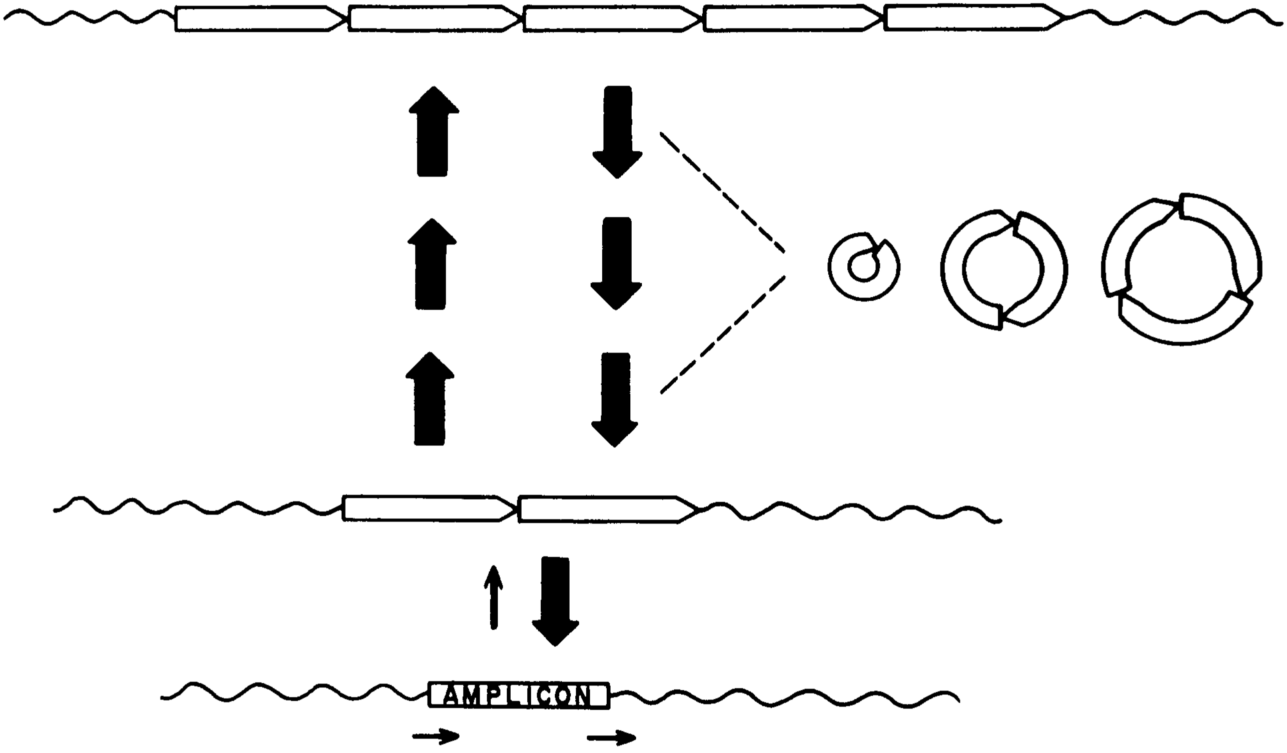

Fig 1. Dynamic model of the *Rhizobium genome* (see text)

that 1 out of 300 cells contains the duplicated region (Brom et al, 1991). Romero et al (1995) have recently reported the dynamic map of the symbiotic plasmid of *R. etli* strain CFN42. In addition to the above described amplicon the map shows the presence of three other major amplicons that duplicate at a frequency higher than 10^{-5}. The four amplicons are overlapping structures and are clustered in a region of the plasmid rich in nodulation and nitrogen fixation genes.

Using the Tn5 GDYN1 element we have detected amplicons in several regions of the genome of *R. etli* strain CFN42, including the chromosome and several large plasmids (Flores et al, 1993). Amplicons varied in size from 10 to more than 100 kb. As mentioned above, amplified regions liberate complete amplicon sequences as closed circular structures. We have developed an experimental strategy for the recovery of such sequences as stable plasmids in *E. coli* (Flores et al, 1993). This *in vivo* cloning procedure can be a valuable tool for studies on genome organization and function.

BIOLOGICAL SIGNIFICANCE OF GENE AMPLIFICATION.

In several cases gene amplification has been correlated with adaptative phenomena in bacteria: growth in poor carbon sources in *E. coli* (Langridge 1969; Neuberger y Hartley, 1981), increased growth in limiting carbon sources in *Salmonella* (Sonti, Roth, 1989), utilization of exotic carbon compounds in *Pseudomonas* (Ghosal et al, 1985), increased virulence in *Vibrio colera* (Mekalanos, 1983) and in *Haemophilus influenzae* (Corn et al, 1993), increased antibiotic resistance in different bacteria (Peterson and Rownd, 1985), to mention some of them.

Gene amplification might be of particular relevance in *Rhizobium* in regard to its adaptation to changing environmental conditions or to its interaction with different plants. Romero et al (this volume) have recently found that genomic rearrangements, including gene amplification, may lead to increased nitrogen fixation in *Rhizobium etli.*

The above mentioned data clearly show that under certain conditions gene amplification has an important biological role for different bacteria. Since the amplified state is a transient reversible condition, gene amplification increases the potentiality of the genome without disrupting its structure. In the context of the model discussed in this paper, each genome presents a specific dynamic structure. Such structure is a result of the position of DNA sequences that may be used as end points for homologous recombination.

Is the amplicon structure of the genome subjected to selection during evolution? Should the amplicon be considered as a genomic unit that coordinates sets of adjacent genes? Does the set of transcriptional units present in an amplicon correspond to a coherent biological function? It is our view that these questions must be addressed in order to gain insight in regard to the biological significance of gene amplification.

ACKNOWLEDGEMENTS

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DEVELOPMENTAL REGULATION OF NITROGEN FIXATION GENE EXPRESSION IN *RHIZOBIUM MELILOTI*-ALFALFA SYMBIOSIS

SOUPÈNE E., FOUSSARD M., TRUCHET G., BOISTARD P.
AND J. BATUT
Laboratoire de Biologie Moléculaire des Relations Plantes-Microorganismes.
CNRS-INRA, BP 27, 31326 Castanet-Tolosan Cédex, France.

INTRODUCTION

The legume nodule is an interesting model for plant developmental biology. Nodules are genuine organs, specialized in nitrogen fixation, that originate from the coordinated differentiation of a bacterial and a plant partner. The molecular dialogue taking place during the early stages of symbiotic interaction and leading to nodule organogenesis, has been largely deciphered in the last few years (see Dénarié, Cullimore 1993 for a review). By contrast, signal exchanges occuring at later stages of the symbiotic interaction and resulting in a fully differentiated nitrogen-fixing nodule are still unknown.

The alfalfa nodule is an appropriate material for such developmental studies since, as a consequence of its permanent growth from an apical meristem, "bacterial" and plant tissues at successive stages of differentiation can be visualized along the longitudinal axis of a mature nodule. We and others have observed that several key symbiotic genes, of either plant or bacterial origin, display a similar pattern of expression *in planta* (de Billy et al. 1991, Franssen et al. 1992, Kardailsky et al. 1993). These include bacterial *nif* and *fix* genes and late nodulins. Late symbiotic genes are silent in zone II, the prefixing zone of the nodule where bacteria invade the plant cells (Figure 1A). In contrast, expression of these genes is turned on abruptly at a region of the nodule called interzone II-III that precedes the nitrogen fixing zone III (Vasse et al. 1990). These observations pointed to interzone II-III as a landmark in nodule differentiation. As a starting point towards the identification of the signal(s) responsible for this developmental regulation, we have recently investigated the regulation of two key regulatory nitrogen fixation genes, *nifA* and *fixK*, *in planta*.

RESULTS AND DISCUSSION

1. Oxygen patterns *nifA* and *fixK* expression *in planta*

Since former studies have established the prominent role of oxygen in controlling nitrogen-fixation gene expression *ex planta* (Ditta et al. 1987, David et al. 1988), we hypothesized that oxygen could determine the pattern of *nifA* and *fixK* gene expression *in planta* as well. Two complementary approaches were taken to test this possibility.

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The first, physiological, approach consisted of manipulating oxygen concentration inside the nodule. This was simply achieved by embedding mature (3-week old) nitrogen-fixing nodules in soft agar overnight. This treatment resulted in a sharp decrease in oxygen concentration inside the nodules whilst not having any visible effect on nodule histology. In particular, interzone II-III was unchanged. Remarkably, ectopic expression of both *nifA* and *fixK* was induced in zone II in treated nodules (Figure 1B and data not shown).

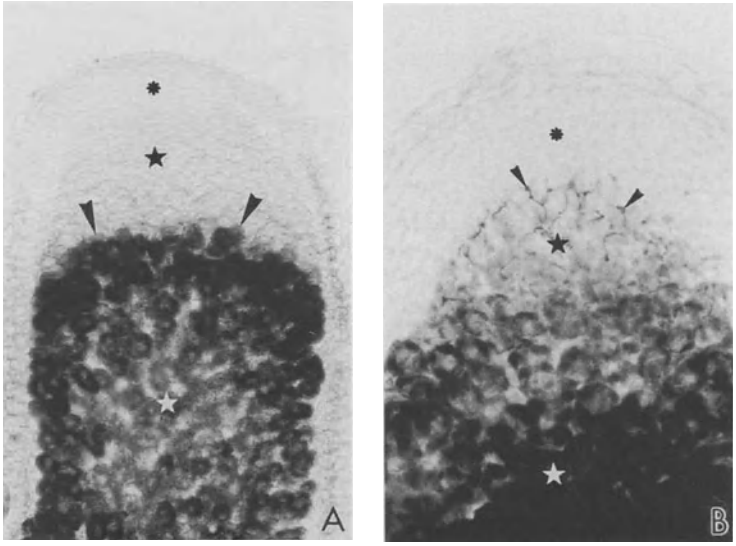

Figure 1: Effect of the nodule gaseous environment on *R. meliloti fixK* gene expression. (A) Control nodule (B) Nodule submitted to a microoxic environment. Asteriks, nodule meristem; black stars, prefixing zone II; large arrowheads, interzone II-III, white stars, N_2-fixing zone III; small arrowheads, infection threads.

In order to ensure that the effect described above was specifically due to a change in oxygen concentration, we chose a genetic approach. We investigated the pattern of expression of *nifA* and *fixK* genes in nodules induced by a *R. meliloti* mutant strain that expressed these genes constitutively with respect to oxygen *ex planta*. The rationale for engineering this mutant is as follows. In *R. meliloti, nifA* and *fixK* expression is under the direct control of a two-component regulatory system encoded by the *fixLJ* operon (David et al. 1988). *fixL* codes for an hemoprotein kinase (Gilles-Gonzalez et al. 1991) that, under microoxic conditions, autophosphorylates and phosphorylates the FixJ transcriptional activator, thereby increasing its activity at the *nifA* and *fixK* promoters (Lois et al., 1993; Reyrat et al. 1993; Gilles-Gonzalez, Gonzalez 1993). We have discovered recently (Reyrat et al. 1994) that a mutant FixJ protein, in which the usual site of phosphorylation, aspartate 54, had been changed for an asparagine, could still be activated by FixL in an oxygen-independent fashion. These observations, mainly obtained *in vitro* with purifed FixL and FixJ proteins, prompted us to introduce the corresponding single point mutation into the *R. meliloti* genome. This was realized by means of a new procedure that allowed us to introduce the unmarked point mutation at the homologous locus. Expression of *nifA* and *fixK* genes in the *R. meliloti fixJD54N* mutant was studied both *ex planta* and *in planta*.

Expression of *nifA* and *fixK* in the mutant was found to be independent of the oxygen status of the culture under free-living conditions,thus confirming the observations made *in vitro* (Figure 2). *In planta*, both *nifA* and *fixK* expressed in zone II of nodules grown under normal ("aerobic") conditions. Hence a single point mutation that derepresses expression of *nifA* and *fixK* under free-living aerobic conditions also derepresses their expression in zone II of the nodule.

Taken together, these two lines of evidence suggest that oxygen is one of the signals acting at interzone II-III that patterns nitrogen fixation gene expression.

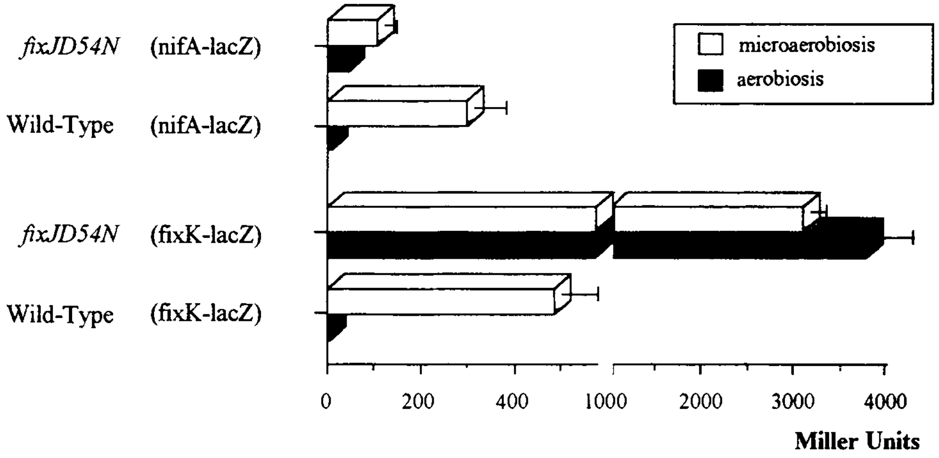

Figure 2: Expression of *nifA*- and *fixK-lacZ* fusions in a *R. meliloti fixJD54N* mutant and in the isogenic wild-type strain in free-living bacterial cultures.

2. Oxygen distribution in the distal part of the nodule

As the data pointed to a key role of oxygen in patterning N_2-fixation gene expression, we attempted to directly investigate oxygen distribution in the distal part of the alfalfa nodule. Our approach consisted in stepwise insertion of an O_2-sensitive micro-electrode along the longitudinal axis of the nodule, from the apex towards the interzone II-III. We found that oxygen concentration progressively decreased from 250 µM, the free O_2 concentration in air-saturated water, to the limit of detection of the micro-electrode (*ca.* 1 µM) at interzone II-III (Figure 3).

Although the decrease of oxygen concentration in zone II is consistent with a signalling role of oxygen, its gradual nature does not readily account for the abrupt upshift in nitrogen fixation gene expression observed at interzone II-III. We propose at least three explanations for this observation.

First, oxygen concentration may actually undergo a dramatic decrease at the interzone II-III boundary that the limited sensitivity of the microelectrode (*ca.* 1μM) did not allow us to detect. Indeed, oxygen concentration in the central tissues of the nitrogen-fixing zone of the nodule is in the 10 to 30 nM range as measured by leghemoglobin spectroscopy experiments (Layzell et al. 1990). Thus oxygen concentration could decrease by 50 fold at the interzone II-III boundary, thereby eliciting a sudden increase in *nif* gene expression. Although we cannot rule out this possibility, it is not consistent with the fact that *nifA* and *fixK* expression apparently takes place at several μM of O_2 under free-living conditions.

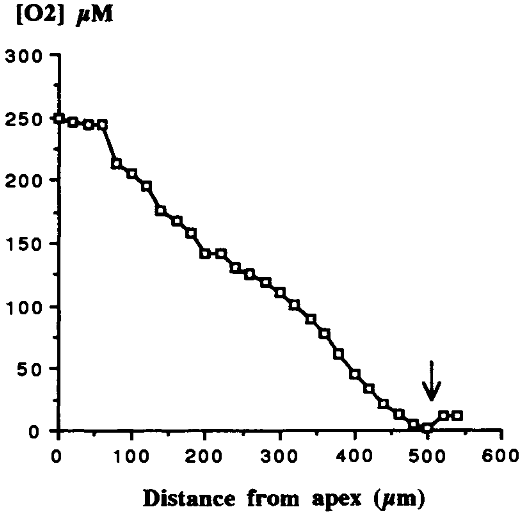

Figure 3: Oxygen gradient profile in the distal part of a mature alfalfa nodule. The arrow marks the position of interzone II-III.

Second and alternatively, *nifA* and *fixK* could respond sharply to a moderate change in oxygen concentration at the interzone II-III boundary. It has been shown in animal systems that monotonic gradients of morphogens can determine discrete patterns of gene expression (Green, Smith 1991). We note that the pathway leading from oxygen sensing by FixL to activation of target *nifA* and *fixK* promoters by phosphorylated FixJ is a multistep process. Oxygen, upon binding to FixL, limits its capacity for autophosphorylation, and thus its capacity to phosphorylate FixJ, and instead enhances its phosphatase activity leading to FixJ-phosphate dephosphorylation (Lois et al. 1993; Gilles-Gonzalez, Gonzalez 1993. Phosphorylated FixJ selectively binds DNA, possibly in an oligomeric form (Galinier et al. 1994). Clearly, there is the potential that the signal generated by a small decrease in oxygen concentration is amplified, causing a large change in gene expression. In addition, microoxy may act at more than one level and, for example, induce the production of a plant compound that would potentiate the bacteroid response *via* the FixL/J system.

Finally, the pattern of expression of *nifA* and *fixK* may not be solely under oxygen control, *i.e.* another mode of regulation would superimpose on that exerted by oxygen. However, the fact that a point mutation in the oxygen responsive regulatory protein FixJ alters the pattern of expression of nitrogen fixation genes during symbiosis indicates that perception of any putative additional regulatory signal of *nifA* and *fixK* must be mediated through the FixLJ regulatory pathway.

3. Is oxygen the only signal operating at interzone II-III?

Whether other signals are operating independently of, or synergistically with, oxygen at the interzone II-III boundary remains an open question. Several lines of evidence tend to support this possibility. First, as we mentioned above, the pattern of oxygen distribution in the distal part of the nodule does not immediately account for the abrupt induction of *nifA* and *fixK* gene expression at interzone II-III. Second, whereas the *fixK*-regulated *fixN* gene expression is identical to that of *fixK* and *nifA*, expression of *nifA*-dependent genes such as *nifHDK*, the structural genes for nitrogenase, appears to be more tightly controlled. So far, we have been unable to trigger ectopic expression of *nifH* in zone II, even in nodules put under severe anoxic conditions. Although the possibility remains that this tight regulation of *nifH* is due to an exquisite sensitivity of NifA to oxygen (Beynon et al. 1988; Fischer 1994), the alternative possibility is that a, so far unknown, signal acts in conjunction with oxygen to control *nifH* expression *in planta*. Third, as we mentioned in the introduction, other symbiotic genes, besides nitrogen fixation genes, are also switched on at interzone II-III. This is particularly the case of the late nodulin, leghemoglobin (de Billy et al. 1991). We have found, using *in situ* hybridization, that the pattern of expression of leghemoglobin could not be modified by manipulating the oxygen environment of the nodule.

Altogether these observations suggest that oxygen may not be the unique signal operating at interzone II-III.

CONCLUSION

Nitrogen fixation is always under tight physiological control. One recurrent regulatory molecule in nitrogen fixers, either free-living or symbiotic, is oxygen. The universal implication of oxygen regulation in nitrogen fixation originates from the fact that nitrogenase is oxygen sensitive. There is thus a need to prevent spurious nitrogenase synthesis in the presence of high oxygen. In addition, nitrogen fixation in the symbiotic state requires a coordinated differentiation of the plant and bacterial partners. Hence nitrogen fixation in *Rhizobium* is under a dual, physiological and developmental, control. So far the relationship between these two types of control was uncertain. Our data strongly suggest that a single signal molecule, oxygen, that was known from previous work to have a key role in controlling nitrogen fixation gene expression in *Rhizobium* is also involved in the developmental regulation of these genes. Thus low oxygen concentration may couple nitrogen fixation gene expression to nodule differentiation and, at the same time, fulfil an essential requirement for nitrogenase activity. We anticipate that other signal molecules exist *in planta* that are involved in either physiological or developmental control, or both. As we mentioned above, several observations suggest that more than one developmental signal is acting at interzone II-III. In addition, since the ultimate role of symbiotic nitrogen fixation -which is highly energy consuming- is to fulfil the plant nitrogen requirements (and not that of the bacterium as in free-living diazotrophs), it is likely that the plant, *via* unknown signals, regulates the nitrogen fixation activity of its microsymbiont in response to its metabolic status. Identifying such signals will be the task of our future studies. One potential difficulty is that these signals, contrary to oxygen, may be plant compounds exclusively synthesized under symbiotic conditions. Nevertheless we hope that a combined genetic, physiological and biochemical approach should made possible the identification of these signals.

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NODULATION GENES OF *RHIZOBIUM GALEGAE*

LINDSTROM, K.[1], PAULIN, L.[2], ROOS, C.[2] AND SUOMINEN, L.[1]
[1]Department of Applied Chemistry and Microbiology, P.O. Box 27, and [2]Institute of Biotechnology, P.O. Box 45, FIN-00014 University of Helsinki, Finland

Rhizobium galegae infects goat's rue (*Galega orientalis* and *G. officinalis*) in a very host specific manner. Nodules with an apical meristem are formed. *R. galegae* strains only form effective (nitrogen-fixing) nodules on their original host. Strains from *G. orientalis* infect *G. officinalis*, and give rise to almost fully developed nodules which fail to fix nitrogen, and *vice versa* (Lindstrom, 1989). *G. orientalis* is a new, perennial forage legume for Northern conditions. It is very persistent, resistant to disease and suitable for making silage. The plant and its rhizobia originate in the Caucasus and the plant requires inoculation with compatible rhizobia to grow well. Our laboratory has focused on the ecology of nitrogen fixation, the taxonomy of *R. galegae*, molecular biology of the interaction between symbionts and the development and application of molecular tools for identification and classification of the rhizobia. *R. galegae* is the only *Rhizobium* species described so far that belongs to the same phylogenetic branch as *Agrobacterium vitis, A. rubi* and biovar 1 (*A. tumefaciens* and *A. radiobacter*, Fig. 1). We are interested in the basis for the host specificity in our system and in the evolution of nodulation functions. This report describes the phylogeny of common nodulation genes and *nodD* genes of *R. galegae* and the identification of another nodulation gene region.

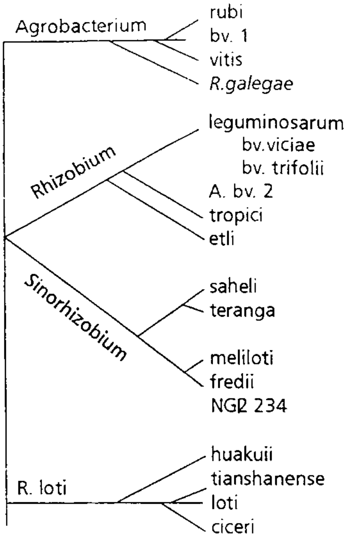

Figure 1. A schematic phylogenetic tree of fast-growing rhizobia drawn after references Chen et al. (1995), deLajudie et al. (1994), Nour et al. (1994), Sawada et al. (1993), Segovia et al. (1993) Willems, Collins (1993), Yanagi, Yamasato (1993) and unpublished results.

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MATERIALS AND METHODS

Cloning of common nodulation genes was done by complementing a nodulation deficient mutant of *R. meliloti* (Suominen et al., 1995a) with a cosmid library of genomic DNA from *R. galegae* HAMBI1174. Clone pRg30 was isolated and the identity of the common *nod* region was verified by hybridization and complementation of *nodA, B* and *C* mutants (Rasanen et al., 1991, Suominen et al., 1995a). Putative genes for host specificity were cloned by probing a cosmid library with a synthetic *nod* box probe. This approach yielded the common *nod* region plus overlapping clones spanning a region with three additional *nod* boxes, all of which are carried by clone pRg10 (Suominen et al., 1995b). Different sized fragments from the common *nod* and host gene regions of *R. galegae* HAMBI1174 were sequenced by Sanger's dideoxy method using the AutoRead Kit (Pharmacia, Uppsala, Sweden) and the sequencing reactions were run on an Automated DNA Sequencer A.L.F. (Pharmacia). A nested set of deletions of the larger fragments to be sequenced was done using the Nested Deletion Kit (Pharmacia). The other sequences were gathered from databases (Genebank and EMBL). Phylogenetic trees were constructed from translated sequences (Swissprot) after sequence alignment from Kimura-corrected distances using the neighbour-joining method. Bootstrap scores were calculated from 100 samples (200 for the partial *nodC*).

RESULTS

Common nodulating genes. Sequencing of the 5.7 kb fragment revealed open reading frames corresponding to *nodD, A,B,C,I* and *J* of other rhizobia. The *nodABCIJ* genes are preceded by a *nod* box 196 bp upstream of *nodA*. *nodD (nodD1)* is transcribed in opposite direction. Phylogenetic trees were constructed for *nodA,B,C* and *D1* after alignment of the translated products of *nod* gene sequences available in the databases (Fig. 2). The *nodABC* genes of *R. galegae* are closest related to the corresponding genes from *R. leguminosarum bv. viciae* and *trifolii* and of *R. meliloti*.

Genes for host specificity. The three *nod* boxes on pRg10 represent strong evidence for the presence of plant-induced genes (Suominen et al., 1995b, see Suominen et al., this volume for comparisons of *nod* boxes). A *nodD* homolog, called *nodD2*, was identified by hybridization and sequencing on pRg10 and hybridization of pRg10 with a 6.8 kb *hsn* fragment with *nodHFEG* from *R. meliloti* 41 gave a hardly detectable signal with a 8.6 kb *Eco*R1 fragment at low stringency. A 2.4 kb fragment was found to be species specific and has subsequently been developed for diagnostic purposes (Tas et al., 1995). The sequence was monomorphic among *R. galegae* strains in Southern hybridizations with *Eco*R1, *Hind*III and *Bam*HI digested DNA (Radeva et al., 1995), but polymorphism was detected after PCR-RFLP of a subfragment, which separated strains from *G. orientalis* and *G. officinalis*. The 2.4 kb fragment is a strong candidate for host specificity genes. Sequencing of the 3.8 kb fragment which carries *nodD2* did not reveal the presence of genes with homology to sequences in databases.

***nodD* genes.** *nodD1* is activated by the synthetic flavonoid, apigenin and by apigenin-7-0-glucoside and luteolin-7-0-glucoside from *Galega* root exudate. The activity of a so far unidentified HPLC fraction surpasses the other inducers and this fraction is supposed to carry the main inducers(s) of *R. galegae nod* genes. The activity of *noD2* is unaffected by plant exudate, but it shows a weak constitutive *nodA* inducing activity in *lac*-fusion tests (Suominen et al., 1995c). The phylogeny of *nodD1* resembles that of *nodABC* (Fig. 2).

DISCUSSION

Because of its phylogenetic position among agrobacteria, *R. galegae* offers an interesting possibility to study the evolution of rhizobia and their nodulation functions. The role of *nod* genes in the construction of the signal to the plant, the Nod factor, has recently been the subject of several reviews (e.g. Carlson et al., 1994, Pueppke, 1995, Van Rijn, Vanderleyden, 1994). The evolution of bacterial genomes have several alternative routes to take: random and adaptive mutations, recombination, lateral gene transfer, and gene duplication. Guttman and Dykhuizen (1994) estimated that in four

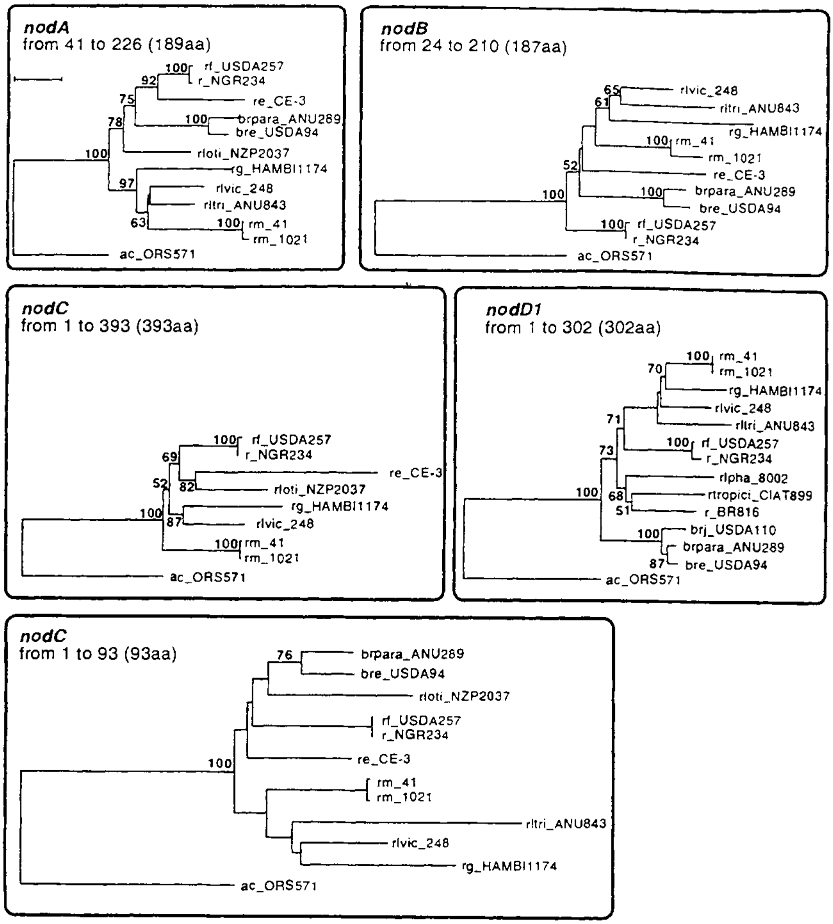
nodA
from 41 to 226 (189aa)
100
92
75
78
100
97
63
rf_USDA257
r_NGR234
re_CE-3
brpara_ANU289
bre_USDA94
rloti_NZP2037
rg_HAMBI1174
rlvic_248
rltri_ANU843
rm_41
rm_1021
ac_ORS571
nodB
from 24 to 210 (187aa)
65
61
52
100
rlvic_248
rltri_ANU843
rg_HAMBI1174
rm_41
rm_1021
re_CE-3
brpara_ANU289
bre_USDA94
rf_USDA257
r_NGR234
ac_ORS571
nodC
from 1 to 393 (393aa)
100
69
52
82
100
87
rf_USDA257
r_NGR234
re_CE-3
rloti_NZP2037
rg_HAMBI1174
rlvic_248
rm_41
rm_1021
ac_ORS571
nodD1
from 1 to 302 (302aa)
100
70
71
73
100
68
51
100
87
rm_41
rm_1021
rg_HAMBI1174
rlvic_248
rltri_ANU843
rf_USDA257
r_NGR234
rlpha_8002
rtropici_CIAT899
r_BR816
brj_USDA110
brpara_ANU289
bre_USDA94
ac_ORS571
nodC
from 1 to 93 (93aa)
76
100
brpara_ANU289
bre_USDA94
rloti_NZP2037
rf_USDA257
r_NGR234
re_CE-3
rm_41
rm_1021
rltri_ANU843
rlvic_248
rg_HAMBI1174
ac_ORS571

Figure 2. Phylogenetic trees of nod*A*, *B*, *C* and *D1* based on translated sequences. Only bootstrap scores above 50% are indicated. rg_HAMBI1174: ***Rhizobium galegae*** HAMBI1174; rlvic_248: ***R. leguminosarum*** bv. ***viciae*** 248; rl_tri_ANU843; ***R. leguminosarum*** bv. ***trifolii*** ANU843; rlpha_8002: ***R. leguminosarum*** bv. ***phaseoli*** 8002; re_CE-3: ***R. etli*** CE-3; rtopici_CIAT899; ***R. tropici*** CIAT899; rm_1021: ***Sinorhizobium meliloti*** 1021; rm_41: ***S. meliloti*** 41; rf_USDA257: ***S. fredii*** USDA257; r_NGR234: ***Sinorhizobium*** sp. NGR234; r_BR816: ***Sinorhizobium*** sp. BR816; rloti_NZP2037: ***R. loti*** NZP2037; brj_USDA110: ***Bradyrhizobium japonicum*** USDA110; bre_USDA94: ***B. elkanii*** USDA94; br_ANU289: ***Bradyrhizobium*** sp. (***Parasponia***) ANU289; ac_ORS571: ***Azorhizobium caulinodans*** ORS571.

E. coli genes a greater proportion of genetic variation had arisen through recombination than through mutation, and Labedan and Riley (1995) found that almost half of the protein coding genes in *E. coli* had evolved *via* gene duplication. It is today generally accepted that lateral gene transfer is involved in the evolution of rhizobial nodulation functions (Dobert et al., 1994, Kaijalainen, Lindström, 1989, Sprent, Raven, 1992, Sprent 1993, Young, Johnston, 1989).

The phylogenetic trees obtained in this study show that *nodABC* of *R. galegae* is most closely related to the corresponding genes from *R. leguminosarum* biovars *viciae* and *trifolii* and of *S. meliloti*. Thus, the *R. galegae nodD1ABC* are the closest related to genes from rhizobia that nodulate temperate legumes and have a narrow host range. In all rhizobia the two halves of NodA are different in that one domain is very conserved whereas the other one shows more dissimilarities. A dendrogram constructed using the conserved domain of NodA placed the *R. galegae* sequence within *R.leguminosarum*, whereas the tree obtained by using the less conserved domain placed *R. galegae* outside *R. leguminosarum* and *S. meliloti*.The distances in the latter tree were much longer than in the former, which indicates more rapid evolution under less constraint (Suominen et al., 1995a). It will be interesting to see whether *nodFE* will also be found in *R. gaelgae*, or whether it has developed a different way to express host specificity than through a multisaturated fatty acid substituent on its Nod factor.

In Fig. 2 *R. etli* is anomalous in that its *nodA* and part of *nodB* group with strains that nodulate soybean, the distal part of *nodB* with *S. meliloti* and *nodC* with *R. loti* (Suominen et al., 1995a).The anomaly might be a reflection of the organization of its *nod* genes, *nodA* is separated from *nodBCIJ* (Table 1).

Recently, *nodSU* have been discovered in many tropical rhizobia (Table 1). *nodS* has been invoked as needed for nodulation of *Leucaena* and acts as a methyl transeferase. It is remarkable that the *nodSU* genes are fairly well conserved among different species (Krishnan et al., 1992, Waelkens et al., 1995) and often organized in one operon together with common *nod* genes, e.g. *nodABCSUIJ* in *A.caulinodans* (Table 1). Whereas *nodFE* is involved in the formation of indeterminate nodules, *nodSU* can be involved in both determinate and indeterminate nodulation. Hybridization with a *nodSU* probe and further sequencing of pRg30 and pRg10 should reveal whether these genes are present in the *R.galegae*. However, they do not reside downstream of *nodC* as in azorhizobia, bradyrhizobia and *R. tropici*. It is interesting to note that *nodSU* are present in *S. fredii* strain USDA257, but are not functional. In strain NGR234 they function, but in neither case are they part of the common *nod* operon. *nodE* homologs have been detected by sequencing in *R.tropici* CIAT899 and in strain BR816 (Table 1).

Another widespread, newly discovered gene is *nodZ* (Table 1). In *A. caulinodans* it lies downstream of *nodJ*. *nodDABCSUIJZ* might represent a nodulation gene package that has been laterally transferred during the evolution. Deletions and rearrangements have certainly occurred since, and additional nodulation genes with no or few counterparts in other species might have evolved *via* gene duplication which probably also contributed to the evolution of several *nodD* copies in the same strain. Both rhizobial species and *nod* gene diversity is greater in the tropics than in temperate regions. According to Woese (1987), bacteria are likely to have higher mutation rates under stressful conditions, which might generate a higher degree of diversity. Under more peaceful conditions mutation rates are lower and fine tuning of life is possible. Since the rhizobia have to adapt to a life in soil and inside the plant, it is difficult to judge whether strict host specificity is beneficial to the symbiotic system. If we accept Woese's hypothesis, fine tuning of life could mean narrow host specificity, which has often evolved in the temperate parts of the world. New species of rhizobia are described at increasing rate. The *Sinorhizobium* and *R. loti* branches have recently got new additions and the

Table 1. Phylogeny of rhizobia and organization of nodulation genes

Phylogenetic branch/ Genus	Species	Strain	Nod gene organization	Host range	Nodule type	Reference(s)
Agrobacterium/ Rhizobium galegae	galegae	HAMBI1174	..D2.,D1ABCIJ'........	N	ID	Lindström, 1989, Suominen, 1995a,b
Rhizobium	leguminosarum bv. viciae	248/pRL1JI	..EF...DABCIJ..........	N	ID	Pueppke, 1995, Spaink et al., 1995
	leguminosarum bv. trifolii	ANU843	..EF...DABCIJ..........	N	ID	Pueppke, 1995, Spaink et al., 1995, Surin et al., 1990
		TA1	...D2....D1.................	N	ID	
	bv. phaseoli	8002	.......D3,D2....BC..D1........	N	D	Davis, Johnston, 1990, Young, Johnston, 1989
	etli	CE-3	........A....BC.IJ..D....	N	D	Segovia et al., 1993, Vázquez et al., 1991, 1993
	tropici B	CIAT899	..D2,D3,D4,D5..D1ABCSU..E..	B	D/ID	Krishnan et al., 1992, Martínez-Romero et al., 1991, van Rhijn et al., 1993, Waelkens et al., 1995
Sinorhizobium	meliloti	Rm1021	..D1ABCIJ(?)..EF.D3..D2..	N	ID	Long 1992
	meliloti	41	..D1ABCIJ..EF..D3..D2.	N	ID	Pueppke, 1995
	fredii	USDA257	..D2..ABC..D1...SU.....	B	D/ID	Pueppke, 1995
	fredii	USDA194	..Z..	?	D/?	Stacey et al., 1994
	sp.	NGR234	..SU...ABCIJ....Z.D1...D2..	B	D/ID	Fellay et al., 1995
	teranga			B	?	de Lajudie et al., 1994
	saheli			B	?	de Lajudie et al., 1994
	sp.	BR816	..D2,D3,D4...D1.E.ABC...	B	D/ID	Martínez-Romero, pers. comm., van Rhijn et al., 1993
Rhizobium loti/ Rhizobium	loti	several	..D3,D2(?),A,BC,IJ,D1,SU,Z..	N(?)	D	Krishnan et al., 1992, Scott et al., database entries, Spaink pers. comm., Young, Johnston, 1989
	huakuii			B(?)	?	Chen et al.,1991
	ciceri			N	?	Nour et al.,1994
	tianshanense			B(?)	?	Chen et al.,1995
Bradyrhizobium	japonicum	USDA110	..D2.D1.YABCSUIJ..Z..	B	D	Luka et al., 1990
	elkanii		..D2.D1.KABC.......	B	D	Dobert et al., 1994
	sp.	ANU289	............DKABC.............	B	D/ID	Becking,1992, Dobert et al., 1994, Scott et al., 1986
Azorhizobium	caulinodans	ORS571	.....DABCSUIJZ.......	B	I,S	Holsters et al. 1994, Ndoye et al.,1994, Waelkens et al., 1995

B: broad host range
N: narrow host range
D: determinate nodules

ID: indeterminate nodules
I: intermediate nodules
S: stem nodules

?: Information not available to author
(?): Uncertain characterization

For host range and nodule type is also referred to Allen and Allen 1981.

Bradyrhizobium certainly carries many more species to be described. It will be interesting to know more about the nodulation properties of those rhizobia and to find out if bradyrhizobia are limited to the tropics and if there are more taxa on the *Azorhizobium* and *Agrobacterium/R. galegae* phylogenetic branches.

For us interested in biodiversity and evolution there are many gaps to fill in Table 1. The distinction between broad and narrow host range is hard to make. Does a rhizobial strain have broad host range if it can nodulate *Phaseolus* and *Leucaena*, or are the plants just promiscous? Pueppke (1995) called for the use of a wider selection of plants when testing nodulation mutants. To that could be added that we need more nodulation studies with newly described species. Host range is a very interesting property of any rhizobia, and we need to check several strains from the different taxa before generalizing about the occurrence and organization of nodulation genes.

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IDENTIFICATION OF GENES, AFFECTING SYMBIOTIC EFFECTIVENESS OF *RHIZOBIUM MELILOTI.*

SHARYPOVA, L.A., SIMAROV, B.V.
Research Institute for Agricultural Microbiology,
Podbelski Shosse 3, St.Petersburg-Pushkin, 189620, Russia.

Introduction.

Inoculation of alfalfa plants with rhizobia allows them to grow even in the absense of combined nitrogen. This ability of rhizobia to increase shoot mass production and nitrogen accumulation is denoted as symbiotic effectiveness (SE). Native rhizobial strains may differ significatnly in the their SE. Moreover, different mutations and recombination events in the *Rhizobium* genome were shown to influence this quantitative trait (for review see Sharypova et al., 1992). SE is interesting not only as a practically important trait, but as an integrate quantitative indicator of a symbiotic system development and function. If we summarize factors, influencing SE, we shall see that they concern all facets of nodule metabolism.

Since nitrogen fixation is an energetically expensive process, it may be limited by the rate of C-substrates supply to endosymbiotic bacteroids. In addition to limitations imposed by the host plant, bacterial symbiont itself plays an active role in making a choice of C-sources through its own transport systems, present in the bacteroid membrane. Only part of C entering bacteroids, is used to support nitrogenase synthesis and function, another part is utilized for the maintainance of basic metabolism and synthesis of reserve materials, such as poly-β-hydroxybutirate (PBM). Therefore, by altering partitioning of C-substrates within bacteroids it is possible to affect either nitrogenase activity or even ultimate productivity of the symbiotic association (Day et al., 1994; Cevallos et al., 1994).

Another group of factors, relevant to the SE is, from the one side, an oxygen concentration within nodules, which is regulated by the host plant, and, from the other side, an energy yield from respiration. The latter may be determined by their ability to recycle H_2, activity of TCA cycle enzymes and efficiency of electron transport chain Evans et al., 1987; O'Brian, Maier, 1989; Day et al., 1994).

The third group of factors is related to the expression of nitrogenase: synthesis of the nitrogen fixation machinery and efficiency of its function. There is no doubt that rate and pathways of ammonia assimilation in plant cells influence productivity of the symbiosis. In particular, modulation of activity of glutamine synthase isoforms by *Pseudomonas* toxin was shown to increase effectiveness of the *R.meliloti*-alfalfa assocoation (Knight, Langston-Unkefer, 1988).

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An influence of any unilateral factor could be negligible, if its action is not met with an adequate response from the symbiotic partner. Therefore, level of integration of plant and bacterial metabolism should be one of the important items, determining effectiveness of the symbiosis.

When we tried to compile genetic data on SE, we found that this trait was related not only to the metabolic exchange between symbiotic partners, but to a broader range of processes. Early reports on *Rhizobium* mutants with improved symbiotic effectiveness, showed that this phenotype was caused by alterations in their basic metabolism (Scherrer, Denarie, 1971; Butvina, 1981; Feodorov, Simarov, 1985) Later, it became possible to link changes in SE with particular steps in symbiosis development and function. Thus, it was found that modification of *R.trifolii nodD* gene by replacement of its part with *R.meliloti nodD*, resulted in increased C_2H_2-reducing activity (Spaink et al., 1989). Some changes in polysaccharides production, were shown to influence SE as well Feodorov et al., 1979; Clover et al., 1989). Currently, it is well established that EPS and LPS play an important role in host cell invasion and development of bacteroids. Therefore, SE is dependent not only on the metabolism of mature bacteroids, but on early steps of bacteroid development.

At the dawn of *Rhizobium* genetics it was beleived that manipulations with *nif*-genes (e. g. amplification of *nif*HDK) would allow to improve effectiveness of *Rhizobium*-legume symbiosis. That veiw underestimated complexity of the symbiotic system and did not take into account limiting factors in nitrogen fixation. In fact, nitrogenase constitutes up to 12% of total bacteroid protein. Regardless of this, there are indications that by interfering in the system of *nif-/fix*-genes regulation, it is possible to increase nitrogenase activity and ultimately - yield of the host plant (Bosworth et al., 1994; Romero et al., 1995).

The largest group of mutations, influencing SE, presumably affect energy supply to nitrogenase. Among them mutations, responsible for the azide-resistant respiration in *R.leguminosarum* and *R.meliloti* (Ram et al., 1978; Butvina et al., 1987), increasing respiration of *R.phaseoli* bacteroids (Soberon et al., 1989), altering C-dicarboxylates transport into *R.meliloti* bacteroids (Engelke et al., 1978), blocking PHB-synthesis in *R.etli* (Cevallos et al., 1994).

It is clear that depending on a symbiotic system, mechanism of SE may be different. The aim of our research was to identify genes responsible for the SE in *R.meliloti*. For this purpose we used 2 approaches: (1) isolating of mutants with increased respiration, (2) isolating of symbiotically improved mutants directly on host plants.

Results

Analysis of *R.meliloti* mutants with increased resperation.

By screening *Tn5*-mutants, generated in *R.meliloti* strain CXM1-188, on plates with 2,3,5-triphenyltetrazolium bromide (TTB), which is an indicator of redox potential, we isolated mutants with increased TTB-reducing activity. They were named Red^{++}. When grown in static culture, the mutants manifested increased production of CO_2. Following inoculation of alfalfa plants 24 out of 28 Red^{++} mutants caused statistically significant increase in N accumulation in shoots. Four Red^{++} mutants were studied for their respiratory activity in the endosymbiotic state. Bacteroids were isolated aerobically in Percoll gradient. Respiration was fueled with succinate and NADH. Bacteroid homogenates, derived from Red^{++} mutants, demonstrated up to 4-fold increase in their

O_2 consumption. We can not be sure, that respiration within nodules, formed by Red^{++} mutants, was really so high, because our measurements were carried out under aerobic conditions. However, the fact, that Red-mutations affected bacteroid respiration, is evident.

Recently, we have started cloning of *red*-genes. By the use of Southern hybridization with *Tn5*-probe, we found, that 23 mutations in the megaplasmid 2 hit at least 14 different genes, and 5 mutations in the chromosome - 5 different genes. Having such a collection of mutants, we hope to get a better insight in the coordination of nitrogen fixation energetic support and host plant growth.

Analysis of *R.meliloti* mutants with increased SE.

Since bacteroid respiration is not the only point, critical to SE, we decided to enlarge our collection of symbiotically improved mutants by screening *Tn5*-mutants directly on host plants. We isolated 27 *R.meliloti* mutants, which caused 15-30% increase in shoot dry mass of inoculated alfalfa plants, as compared to parental strains. Many of them performed better than the parents not only in tube tests, but also in the greenhouse experiment (Fig 1). Interestingly, Eff^{++} mutants did not have significant differences from the parent in their cultural properties and nodulation ability.

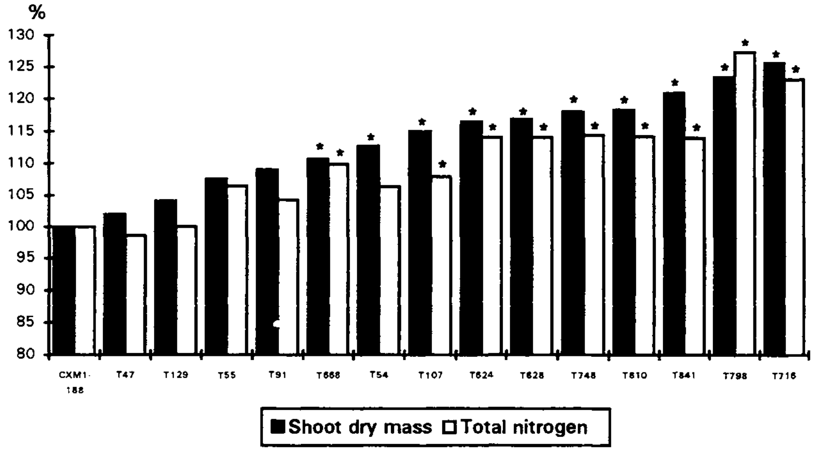
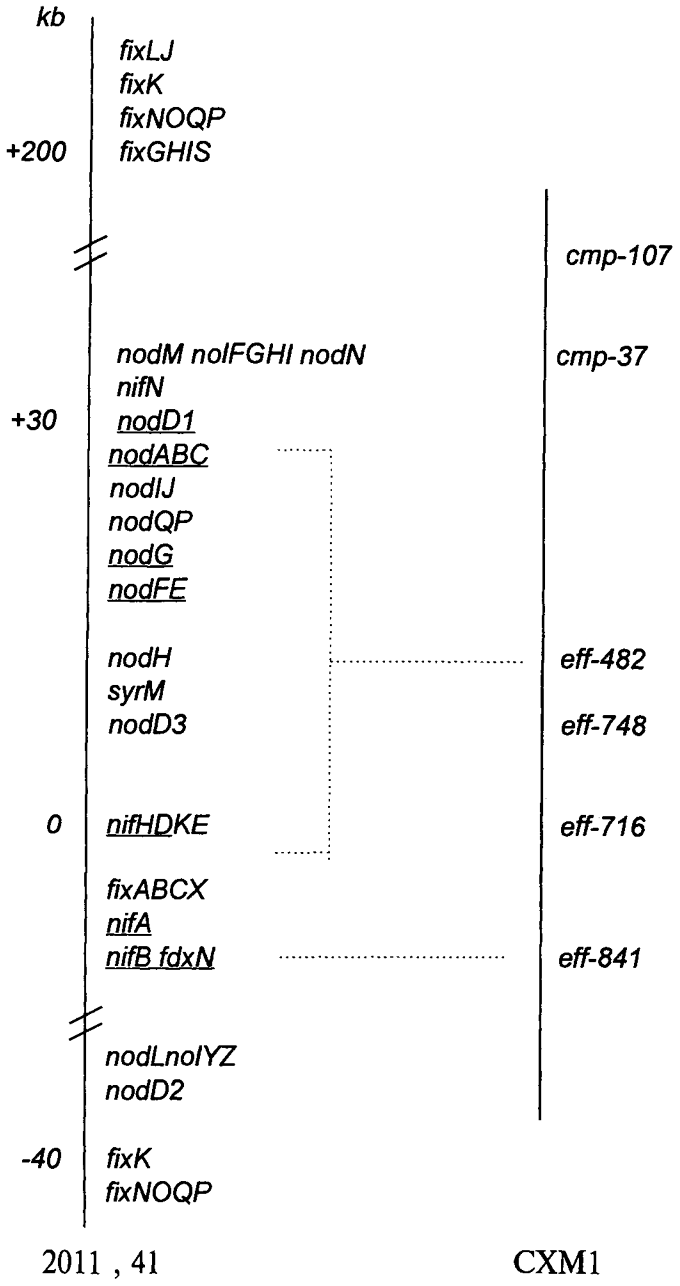


Fig.1. Symbiotic effectiveness of *R.meliloti Tn5* mutants under greenhouse conditions
* - significantly greater than the value for CXM1-188

In contrast to the Red^{++} mutants, which carried *Tn5* preferentially in the megaplasmid 2, Eff^{++} mutants exhibited nearly random distribution of *Tn5* insertions in their genome: 12 in the chromosome, 6 - on the megaplasmid 1 (M1), 9 - on the megaplasmid 2 (M2).

Since some *eff*-mutations were located on the M1, it was tempting to check, whether they hit already known sym-genes. For this purpose, we prepared a number of hybridization probes, carrying *nif*HD, *nif*AB, *fdx*N, *nod*ABCD and *nod*FE-genes, and hy bridized them against genomic DNA of Eff^{++}mutants. None of them showed any alteration in hybridization pattern, as compared to the parent. Therefore, we used general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fixLJ
fixK
fixNOQP
+200
fixGHIS
cmp-107
nodM nolFGHI nodN
cmp-37
nifN
+30
nodD1
nodABC
nodIJ
nodQP
nodG
nodFE
nodH
eff-482
syrM
nodD3
eff-748
0
nifHDKE
eff-716
fixABCX
nifA
nifB fdxN
eff-841
nodLnolYZ
nodD2
-40
fixK
fixNOQP
2011 , 41
CXM1

Fig.2. Genetic map of *R.meliloti* megaplasmid 1
cmp-107 and cmp-37 - loci involved in the genetic control of nodulation competitiveness

transduction to determine positions of *eff*-genes on the genetic map of the M1. We found that 4 *eff*-loci were linked to each other and occupied a region of ca. 35 kb (Fig 2). None of them showed linkage to *fix*G and *nod*ABC, while linkage with *fdx*N was found (a distance from *fdx*N to *eff*-841 was ca. 4 kb, and from *fdx*N to *eff*-716 - ca. 8 kb). When DNA fragment, carrying *eff*-482, was hybridized against genomic DNA of derivatives of strain 41, carrying overlapping deletions in the M1 (Kondorosi et al., 1984), we found that this mutation was located in a region flanked by *nod*ABC and *nif*HDK. Unfortunately we could not determine precise positions of *eff*-loci, since genetic maps of our strain and of reference strains appeared to be non-colinear.

Transductional mapping of *eff*-loci on the M2, revealed that, as in the case of the M1, they were not scattered over the replicon but clustered in one region (Fig.3). Interestingly, 4 *eff*-loci were mapped to a region of *bhb*- and *pca*-loci, responsible for the utilization of poly-β-hydroxybutyrate and protocatechuate (Charles, Finan, 1990).
None of the Eff^{++} mutants was affected in the ability to grow on β-hydroxybutyrate as a sole carbon source. Utilization of protocatechuate was not studied.

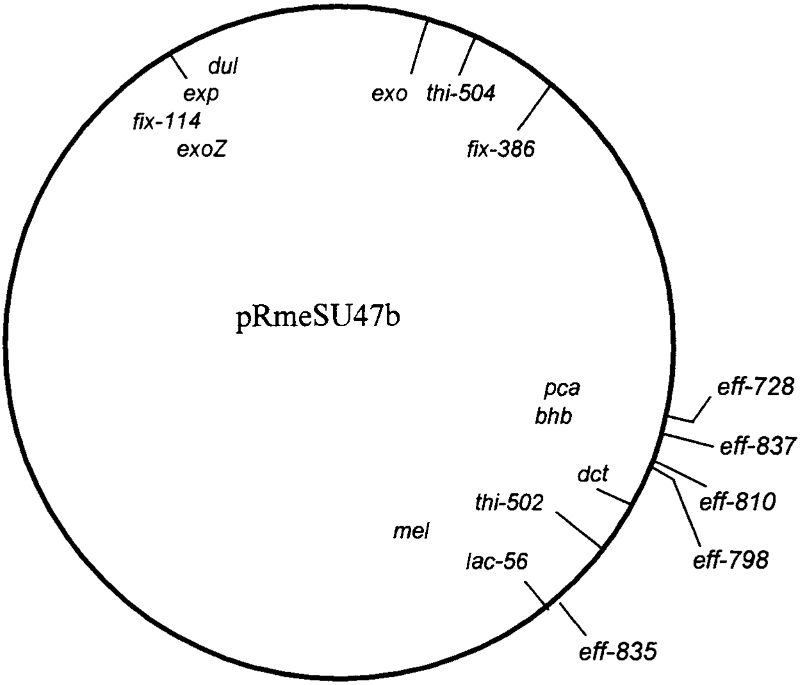

Fig.3. Genetic map of *R.meliloti* megaplasmid 2

We have cloned a number of *eff*-genes and used them as hybridization probes against genomic DNA of different *Rhizobiaceae* species (*R.leguminosarum bv. trifolii, R.l.bv. viciae, R.galegae, R.fredii, A.tumefaciens, B.japonicum*). We could not find any interspecies homology for *eff*-482, *eff*-798 and *eff*-118 (chromosomal locus), while among native *R.meliloti* strains they were well conserved.

To get more information about these *eff*-genes, we initiated their sequencing. Preliminary results from the sequencing analysis evidence that they have no homology to *sym*-genes. For *eff*-482 and *eff*-118 we could not find any homology at the protein level in the data bank. Interestingly, deduced a.a. sequence of *eff*-798 showed siginificant homology with sugar transport proteins (46% identity in 67 a.a. overlap with ribose transport system permease protein of *Bacillus subtilis*, 48% identity in 67 a.a. overlap with homologous protein of *Escherichia coli*, and 45% identity in 62 a.a. overlap with xylose transport permease of *E.coli*).

To conclude, although current knowledge about *sym*-genes of *Rhizobium* seems to be exhaustive, up to now nearly nothing has been known about genetic control of SE - a trait, which reflects coordination of symbiotic partners' developmental programs and integration of their metabolism. We have identified in *R.meliloti* genome more than 30 different genes, responsible for the SE. This will promote study of this complex practically important trait.

Acknowledgements.

We are thankful to our coworkers O.P.Onishchuk, S.N.Yurgel, O.N.Chesnokova, Y.G.Fomina-Eshchenko, E.Sagulenko for their contribution to this work. We have benefited to a great deal from our collaboration with a team of L.A.Syrtsova from the Institute of Chemical Physics RAS (Chernogolovka, Moscow region), and a team of V.N.Ksezenko from the Institute of Biochemistry and Physiology of Microorganisms RAS (Pushchino, Moscow region). The research was supported by Russian programs "Interbioazot" and "Fronties in Genetics".

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GENETIC LOCI INVOLVED IN THE SYNTHESIS AND EXPORT OF RHIZOBIAL LIPOPOLYSACCHARIDES

PRIEFER,U.B., and PRECHEL,H.*
Ökologie des Bodens, RWTH Aachen, 52056 Aachen; * Lehrstuhl für Genetik, Universität Bielefeld, 33501 Bielefeld; Germany

Introduction

The establishment of an effective nitrogen-fixing symbiosis between *Rhizobium* and its host plant requires continuous signal exchange between the two symbiotic partners. It has become substantiated that rhizobial surface carbohydrates, such as the lipopolysaccharide (LPS) and the extracellular polysaccharide (EPS) play a role in this interaction (for reviews see Carlson et al., 1991; Djordjevic et al., 1987; Halverson, Stacey, 1986).

Similar to enterobacterial LPS, the rhizobial LPS has structurally distinct regions: the hydrophobic lipid A, the core oligosaccharide, and a strain specific, immunodominant O-antigen polysaccharide (Bhat et al., 1991; Carlson, 1984; Carlson et al., 1987; Carlson et al., 1989; Carrion et al., 1990; Hollingsworth, Carlson, 1989; Hollingsworth et al., 1989).

Although the chemical composition and structure of LPS molecules of various rhizobial species and strains has been analysed in great detail, only little is known about the genetics of LPS synthesis in *Rhizobium*. Approaches to identify genes involved in LPS production have been initiated by isolating and characterizing transposon-induced LPS mutants. For the species *Rhizobium leguminosarum*, it was found that all mutants defective in the production of an O-antigen containing LPS molecule, were simultaneously impaired in the formation of nitrogen fixing nodules. They either failed to form normal infection threads or showed severe defects in the release and bacteroid formation (Carlson et al., 1987; deMaagd et al., 1989; Noel et al., 1986; Priefer, 1989).

In *Rhizobium leguminosarum* bv. *phaseoli* (strain CFN42), three distinct genetic *lps* regions have been described (Cava et al., 1989; Cava et al., 1990). In *Rhizobium leguminosarum* bv. *viciae*, strain VF39, at least two independent regions important for LPS production and symbiotic efficiency have been identified on the chromosome (Priefer, 1989) and a third one is located on one of the plasmids present in this strain (Hynes, McGregor, 1990). One of the chromosomal *lps* regions could be cloned on a 40-kb DNA insert in cosmid pRlcos4 (Priefer, 1989), which was found to complement the LPS and symbiotic phenotype of three independent Tn5-induced LPS mutants

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(VF39-51, VF39-23, VF39-32). A 10-kb *Eco*RI subfragment, characterized by the mutation in VF39-51, was found to carry at least four independent transcriptional units involved in LPS-O-antigen production (Prechel et al., 1994).
Here, the genetic and molecular analysis of another *lps* region contained on pRlcos4, is described.

Results and Discussion

The chromosomal region of *Rhizobium leguminosarum* bv. *viciae* strain VF39 contained in cosmid pRlcos4, as well as that part described here in more detail, are illustrated in Figure 1.

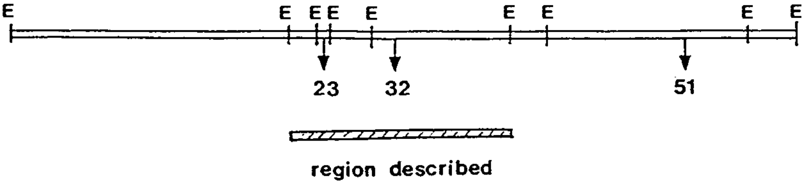

Figure 1: *Eco*RI restriction map of the chromosomal *lps* region cloned in cosmid pRlcos4. The locations of the Tn*5* insertions in the mutants VF39-51, VF39-23, and VF39-32 are indicated by arrows. The region described here in more detail is indicated underneath the physical map.

The area indicated was cloned as overlapping subfragments and sequenced with an automatic laser fluorescence system (A.L.F., Pharmacia). Analysis of the sequence revealed the presence of nine open reading frames (ORFs) with high coding probability (Figure 2).
For some of these open reading frames, similarities with known protein sequences in the data base were found. These results, as well as the data obtained from mutational analyses, are described in the following.

The gene products of the open reading frames, designated ORF2 and ORF3, exhibit similarities to each other and to dTDP-glucose-4,6-dehydratase enzymes from various organisms. Both open reading frames are necessary for the production of a complete, O-antigen containing LPS molecule (LPSI), since LPS preparations from corresponding mutants (ORF2 mutant = VF39-32; ORF3 mutant generated by insertion of an antibiotic resistance cassette) lack the slow migrating LPSI band on

SDS-polyacrylamide gels. Both mutants are also defective in the formation of nitrogen-fixing nodules on *Vicia hirsuta* plants.

For mutant VF39-32, the biochemical analysis, which was published previously, established that this mutant produced a different core oligosaccharide consisting of a trisaccharide instead of the tetrasaccharide present in the wildtype (Zhang et al. 1992). The exact enzymatic functions of these two open reading frames have not been established, so far.

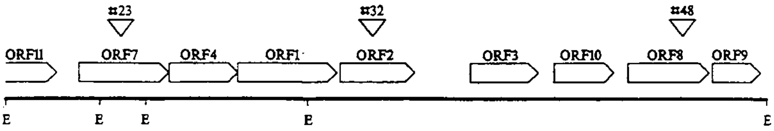

Figure 2: Restriction (only *Eco*RI (E) sites are given) and genetic map of the chromosomal *lps* region described in this study. The position and orientation of open reading frames ORFs), as determined by sequence analysis, are indicated. The location of the original Tn5 insertions in mutants VF39-23 (ORF7) and VF39-32 (ORF2) are given by triangles.

The two open reading frames upstream of ORF2 share similarities to proteins, reported to be involved in the export of polysaccharides. ORF1 shows homology to *E.coli* KpsT and *Haemophilus influenzae* BexA, especially in the region of the putative ATP binding site (Kroll et al., 1988; Pavelka et al., 1991; Smith et al., 1990). ORF4 exhibits similarities to KpsM from *E.coli* and BexB from *H.influenzae* (Kroll et al., 1990; Pavelka et al., 1991; Smith et al., 1990). and is probably a membrane protein since the hydrophobic profile of the ORF4 gene product suggests six membrane spanning domains.

A mutant in ORF1, constructed by insertional mutagenesis, was no longer able to produce an O-antigen containing LPSI, when crude or purified LPS preparations were analyzed by SDS-polyacryl amide gelelectorphoresis. Plant tests on *Vicia hirsuta* revealed, that this mutant generated nodules devoid of nitrogen fixation activity.

To investigate whether the lack of the LPSI moiety was due to the failure of polysaccharide export (as suggested by the protein homology), antibodies against LPSI were raised and used to test the presence of LPSI molecules in membrane and cytoplasmatic cell fractions of wildtype and mutant cells. In a modified sandwich elisa assay, the antibody was bound to the various cell fractions and binding was monitored using an anti-rabbit-antibody labelled with peroxidase. The antibody reacted positively to both, membrane and cytoplasmatic fractions of the wildtype strain, but for the ORF1 mutant, binding of the LPSI antibody was observed only with the cytoplasmatic fraction, not with the membrane preparations. Thus, the mutant appears to be able to synthesize a complete LPSI structure, but is obviously impaired in exporting this molecule to the cell surface.

So far, we have not succeed in inserting an antibiotic resistance cassette into ORF4, which suggests, that mutations in this open reading frame may be lethal.

Also the open reading frame ORF11 (only the C-terminal part is shown in Figure 2) is involved in LPS production. At the amino acid level, ORF11 revealed a significant homology to the amino acid sequence deduced from *Salmonella typhimurium rfbA*, which is involved in the pathway of LPS biosynthesis and encodes a dTDP-glucose-pyrophosphorylase enzyme (Jiang et al., 1991; Reeves, 1993). The ORF11 gene product is also homologous to a similar protein described in Xanthomonas campestris (Köplin et al., 1993).

A mutant in ORF11 was constructed by integrational mutagenesis. This mutant grows very poorly on TY medium, forming very dry and rough, small colonies. Like the other mutants described above, it did not produce an O-antigen containing LPS on SDS-polyacrylamide gels. In addition, the mutant was no longer able to produce measurable quantities of exopolysaccharide.

A plasmid, overexpressing ORF11 under the control of the lac promoter was constructed and introduced into *E.coli*. TDP-glucose-pyrophosphorylase activity was measured and found to be significantly increased (15-20 fold) as compared to the control. Details will be published elsewhere (Prechel et al., in preparation).

All these data strongly suggest, that ORF11 encodes an enzyme involved in a very early step in the formation of a nucleotide sugar, which is essential for both, lipo- and exopolysaccharide production..

Until now, inoculation of *Vicia hirsuta* with the ORF11 mutant has not lead to nodule formation. Therefore, it can be speculated, that this mutant is also impaired in very early steps of symbiosis.

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THE ROLE OF A MICROAEROBICALLY INDUCED *cb*-TYPE CYTOCHROME OXIDASE IN SYMBIOTIC NITROGEN FIXATION

L. THÖNY-MEYER, O. PREISIG, R. ZUFFEREY AND
H. HENNECKE
Mikrobiologisches Institut, Eidgenössische Technische Hochschule,
Schmelzbergstrasse 7, CH-8092 Zürich

INTRODUCTION

How does a root nodule bacterium cope with the problem of meeting the high energy requirements for nitrogen fixation and at the same time protecting the extremely oxygen-sensitive nitrogenase? This "oxygen paradox" (Appleby, 1984) has been one of the central questions in trying to understand how an effective root nodule symbiosis is established and functions. *Bradyrhizobium japonicum* solves this problem by synthesizing a branched respiratory chain with a terminal oxidase that is specifically adapted to microaerobiosis (Hennecke, 1993). Such an oxidase, whose existence has been postulated for a long time (Appleby 1984; O'Brian, Maier 1989; Bergersen, Turner, 1990), is expected to have a high affinity for oxygen, reflected by a K_M in the range of the free oxygen concentration (10-20 nM; Witty, Minchin, 1990) that prevails in root nodules. This enigmatic oxidase should provide a mechanism which guarantees optimal utilization of extremely low oxygen concentrations for energy production.

The discovery of the microaerobically induced *fixNOQP* genes in rhizobia has led to the speculation that they code for the microaerobic oxidase that operates in bacteroids and drives nitrogen fixation. We have investigated the *fixNOQP* genes of *B. japonicum* and biochemically characterized their gene products.

THE *fixNOQP* GENES CODE FOR A MEMBER OF THE HEME-COPPER RESPIRATORY OXIDASE FAMILY

The *B. japonicum* genes *fixNOQP* have been identified in a cluster of genes essential for symbiotic nitrogen fixation (Preisig et al., 1993; see below). The genes were sequenced and amino acid sequence comparisons suggested that FixN is a subunit I homologue of heme-copper oxidases from mitochondria and many different aerobic bacteria (Preisig et al., 1993; García Horsman et al., 1994). The most striking feature FixN shares with other heme-copper oxidase subunits is the conservation of 12 (out of 14) transmembrane-spanning helices with six strictly conserved histidines at specific positions (see references in García Horsman et al., 1994; van der Oost et al., 1994) that

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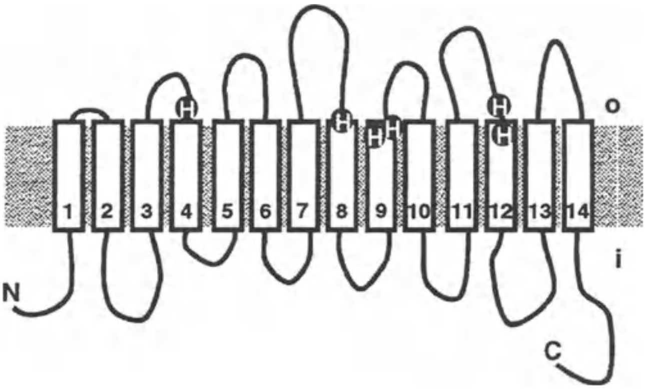
are the axial ligands for a low-spin heme and a high spin heme-Cu_B binuclear centre, where the reduction of molecular oxygen takes place. A topological model of FixN is depicted in Fig. 1. We have experimentally shown that the C terminus of the polypeptide faces the cytoplasmic side of the membrane.

In contrast to classical heme-copper oxidases, there is no evidence for the existence of a subunit II homologue. Instead, the two additionally found subunits, FixO and FixP, are *c*-type cytochromes that are N-terminally anchored to the cytoplasmic membrane. Their bulk domain is believed to be exposed to the periplasm, as it is characteristic for bacterial *c*-type cytochromes (Thöny-Meyer et al., 1994a).

Fig. 1. Topological model of the FixN oxidase subunit I.



FixQ is a small protein (approx. 6 kDa), presumably contains a single membrane-spanning domain and was shown to have a cytoplasmically oriented N terminus, as shown by high-level expression of a translational *lacZ* fusion to the 15th codon of *fixQ*. No other specific motifs were found.

The characteristics of the *fixNOQP*-encoded polypeptides derived from sequence analysis are summarized in Table 1. They point out the unusual composition of this terminal oxidase of the novel cbb_3 type.

Table 1. Characteristics of *the B. japonicum fixNOQP* gene products.

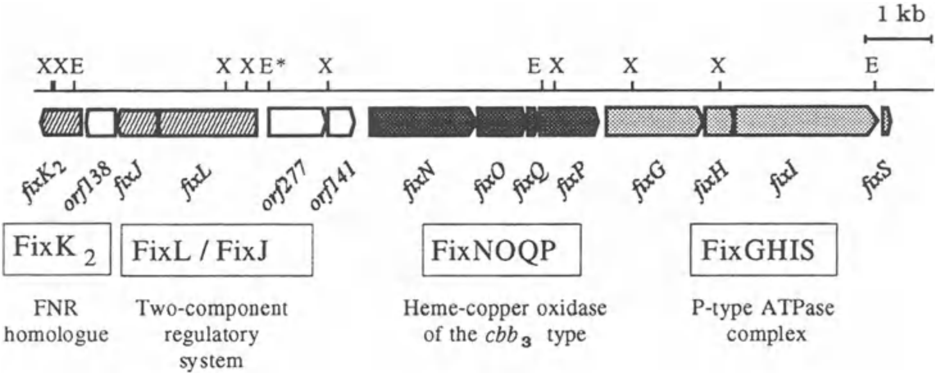
Protein	Amino acids	Calculated M_r (apoprotein)	Localization	Cofactor binding sites
FixN	549	61'272	membrane-integral	2 heme B; Cu_B
FixO	244	27'352	membrane-anchored	heme C
FixQ	54	6'031	membrane-anchored	
FixP	290	31'023	membrane-anchored	2 heme C

The extraordinary combination of a heme-copper oxidase subunit I with two *c*-type cytochrome electron carriers may provide a mechanism for the bacteroids to optimally utilize the low free oxygen in root nodules. Genes homologous to *B. japonicum fixNOQP* have also been identified in other rhizobia (Kahn et al., 1993; Mandon et al., 1994). More recently the presence of *fixNOQP*-like genes was also shown in a wider collection of Gram-negative bacteria, such as *Rhodobacter capsulatus (ccoNOQP), Azotobacter vinelandii, Agrobacterium tumefaciens* and *Pseudomonas aeruginosa* (Thöny-Meyer et al., 1994b; Schlüter et al., 1995). Thus, the presence of this type of oxidase is not restricted to rhizobia. At least from *R. capsulatus* we know that the corresponding *cbb*$_3$-type oxidase can also support aerobic growth. Nevertheless it is likely that the *fixNOQP*-encoded oxidase in rhizobia is functionally specialized for the microaerobic conditions in symbiosis and thus represents the enigmatic bacteroid oxidase, as will be shown below.

REGULATION OF *fixNOQP* GENE EXPRESSION

The *fixNOQP* operon is located in the so-called *fix* cluster III (Fischer, 1994) that contains the regulatory genes *fixLJ* and *fixK*$_2$, the *fixNOQP* operon, the *fixGHIS* operon (O. Preisig, unpublished), and two open reading frames (*orf277* and *orf141*) of unknown function (Fig. 2.).

Fig. 2. The *B. japonicum fix* gene cluster III. In the upper part the physical map of the gene cluster is shown. E: *Eco*RI, E*: *Eco*RI not recognized in DNA isolated from *B. japonicum*, X: *Xho*I. Below, the postulated functions of the gene products are indicated.


1 kb
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E X
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X
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fixK2
orf138
fixJ
fixL
orf277
orf141
fixN
fixO
fixQ
fixP
fixG
fixH
fixI
fixS
FixK 2
FixL / FixJ
FixNOQP
FixGHIS
FNR homologue
Two-component regulatory system
Heme-copper oxidase of the cbb3 type
P-type ATPase complex

The FixLJ two-component regulatory system activates *fixK*$_2$ expression under conditions of low oxygen. FixK$_2$ is homologous to the *E. coli* FNR regulator and activates transcription of the *fixNOQP* and *fixGHIS* operons. We have mapped the transcription start sites in front of these operons and found them at a distance of 41 and 39 nucleotides, respectively, downstream of a conserved consensus element (TTGA----GATCAA) that is thought to represent a FixK$_2$ binding site (Fischer, 1994).

Transcription of both operons is greatly reduced both under aerobic growth conditions and under all conditions in a *fixK*$_2$ mutant background, as shown by *lacZ* expression or transcript mapping (D. Anthamatten and O. Preisig, unpublished). Hence, the *fixNOQP* genes are expressed under microaerobic and anaerobic conditions upon activation by FixK$_2$, and concomitantly with the *fixGHIS* genes.

BIOCHEMICAL CHARACTERZATION OF THE *cbb*$_3$-TYPE OXIDASE

To study the crucial question of oxygen affinity of the *cbb*$_3$-type oxidase we purified it from solubilized membranes of anaerobically grown *B. japonicum* cells. We have shown that the main cytochrome *c* oxidase activity that is found in bacteroid membranes is correlated with the presence of FixO- and FixP-specific *c*-type cytochromes (Preisig et al., in preparation) that are also produced in anaerobically grown cells. Our purified preparation had a 27-fold enriched cytochrome *c* oxidase activity and contained three predominant polypeptides of 42, 27 and 31 kDa, the latter two being heme-stainable. They were predicted to be FixN, FixO and FixP, which was confirmed after isolation of the proteins from an SDS polyacrylamide gel and N-terminal amino acid sequence analysis. No indication for the presence of an additional FixQ subunit in the complex has been obtained thus far.

The spectral characteristics of our oxidase preparation were very similar to those of a previously purified bacteroid oxidase by Keefe and Maier (1993). The only non-covalently bound heme species present in the oxidase was heme B, as shown by heme extraction and HPLC analysis.

Finally, we produced and tested antibodies directed against a MalE'-'FixO and a MalE'-'FixP hybrid protein and showed that they specifically cross-react with the 27 and 31 kDa *c*-type cytochromes of the *cbb*$_3$-type oxidase in microaerobic, anaerobic or bacteroid cells. Antibodies against a mixture of three peptides corresponding to segments of the presumptive cytoplasmic loops 2/3, 10/11 (see Fig. 1) and of the C terminus specifically recognized the FixN subunit. The results from our immunological analyses served as a further indication that the anaerobically produced cytochrome *c* oxidase we have purified is identical with the one expressed in bacteroids.

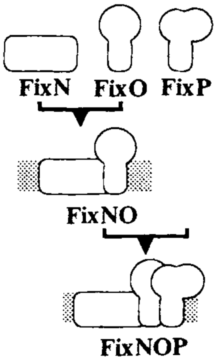
THE *cbb*$_3$-TYPE OXIDASE HAS A HIGH AFFINITY FOR OXYGEN

We first measured oxygen consumption of the purified oxidase, using the leghemoglobin-buffered spectrophotometric assay (Appleby, Bergersen, 1980; D'Mello et al., 1994) providing the very low concentrations of free oxygen that are physiologically relevant for the function of this oxidase. Cytochrome *c* oxidase-dependent deoxygenation of leghemoglobin was measured, and a K_M value of 12 nM O_2 was calculated. This value can be nicely correlated with the average concentration of free oxygen in a root nodule and indeed points to the physiolgical role of the *cbb*$_3$-type oxidase in symbiosis. However, the K_M value must be regarded with caution, because leghemoglobin was partly oxidized during the experiment. This artefact was caused by interaction of leghemoglobin with either cytochrome *c* or ascorbate, both of which served as electron donors for respiration. The only way to circumvent this problem was to measure oxygen consumption of whole membranes. The K_M values for oxygen were determined and compared with membranes of anaerobically grown cells from (i) the wild type, (ii) a Δ*fixNOQP* mutant (see below), and (iii) the COX132 mutant that lacks the *aa*$_3$-type cytochrome *c* oxidase (Bott et al., 1990). In the wild type, a biphasic kinetic behaviour was observed with two K_M values of 19 and 4 nM O_2. This suggests that at least two oxidases with different affinities for oxygen are present in anaerobically grown cells. By contrast, monophasic kinetics were found for both, the COX132 and the Δ*fixNOQP* mutant, with K_M values of 7 nM corresponding to the *cbb*$_3$-type oxidase and 56 nM O_2 corresponding to the *aa*$_3$-type oxidase, respectively. Our results clearly support the hypothesis that the *fixNOQP* genes code for the high-affinity oxidase that is used under microaerobic conditions to gain energy for nitrogen fixation.

ROLE OF THE INDIVIDUAL OXIDASE SUBUNITS

Non-polar in-frame deletion mutations in *fixN*, *fixO*, *fixQ* and *fixP* were created and analyzed for oxidase function and assembly. The constructs were cointegrated into a Δ*fixNOQP* operon deletion mutant and tested for complementation of the phenotype. As a positive control, the operon deletion was also complemented with an intact *fixNOQP* operon. The analysis involved symbiotic nitrogen fixation (Fix phenotype), TMPD- and cytochrome *c* oxidase activities, difference spectroscopy, heme stains and Western blot analysis (Zufferey et al., in preparation).

Fig. 3. Assembly of the *cbb*$_3$-type oxidase subunits into a membrane-integral complex. For details see text.



The results can be summarized as follows: (i) the Δ*fixN*, Δ*fixO* and Δ*fixP*, but not the Δ*fixQ* deletion mutants showed reduced Fix-, TMPD- and cytochrome *c* oxidase activities. This suggests that FixN, FixO and FixP are subunits of the oxidase and are required for its biological function. By contrast, FixQ may be a non-essential subunit of the complex, or it may be used to some extent for assembly or stability of the oxidase. (ii) Spectroscopic analyses showed a significant reduction of *c*- and *b*-type cytochromes in membranes of the three Fix$^-$ mutants, and a marginal reduction in a Δ*fixQ* mutant. (iii) In the Δ*fixN* and Δ*fixO* mutants all three heme-carrying subunits were missing, whereas they were present in the Δ*fixQ* mutant. In the Δ*fixP* mutant FixP was absent whereas FixN and FixO were present. We conclude from these results that for assembly of the oxidase FixN and FixO are initially required to form a core complex, which assembles independently from the presence of FixP (Fig. 3). FixP is subsequently incorporated into the complex, and its persistence in membranes depends on formation of that core complex. FixQ is not essential for oxidase assembly.

THE POSSIBLE ROLE OF THE *fixGHIS* GENE PRODUCTS

We have cloned and sequenced the *B. japonicum fixGHIS* genes (Preisig, Hennecke, in preparation) whose products share 27-58% amino acid sequence identity with the *fixGHIS* gene products of *Rhizobium meliloti* (Kahn et al., 1989). The data suggest that FixI is a P-type ATPase involved in heavy metal transport and FixG, H and S are additional subunits associated with this transport. The coordinated expression with, and the conserved localization downstream of, the *fixNOQP* operon suggested to us that FixGHIS might be involved in assembly of the cbb_3-type oxidase. A Δ*fixGHI* strain was constructed and its phenotype analyzed. The mutant was defective in symbiotic nitrogen fixation (Fix$^-$). Heme stain and Western blot analysis of Δ*fixGHI* mutant membrane proteins revealed that the FixN, FixO and FixP proteins were either absent or present in barely detectable amounts. This finding supports our speculation that the FixGHIS proteins form a membrane protein complex required for maturation of the cbb_3-type oxidase. Possibly, the specific function is transport of copper before it is incorporated into the oxidase.

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THE SYMBIOTIC PLASMID AND NODULATIOIN GENES IN *RHIZOBIUM HUAKUII* CHEN

ZHANG, Z., NONG, G., HU, F., ZHANG, X., ZOU, X., ZHOU, J., LI, F., CHEN, H.
Biotechnology Center, Huazhong Agricultural University, Wuhan, 430070, P. R. China

Introduction

Rhizobium huakuii Chen (Chen et al., 1991) is the rhizobia isolated from nodules of *Astragalus sinicus* L., which forms nodules mainly on *A.sinicus* and a few *Astragalus* spp. in a very narrow host-specific manner (Chen et al., 1944). *A.sinicus* is an important legume crop for green manure, traditioinally grown in rice fields in Yangtze River Valley and southern China. The biology, physiology and ecology of the *A.sinicus R.huakuii* symbiosis, as well as its application in agriculture have been extensively studied for several decades in China (Chen et al., 1992). Studies on its genetics have been conducted only lately. This paper focuses on the identification of symbioitic plasmid and cloning of early symbiotic genes in *R.huakuii*

Indigenous plasmids and symbiotic genes in *R.huakuii*

The plasmid profiles of 154 strains of *R.huakuii* were examined by a modified Eckhardt (1978) procedure of plasmid visulization. They contained 1 to 5 indigenous plasmids and the molecular weight of the plasmids varied from 30 to 600 MD (Zou et al., 1994). The Southern blots of plasmids of effective strains were hybridizated with *nod*DABC (Downie et al., 1983) (from *R.leguminosarum*) and *nif*HDK (Cannon et al., (1979) (from *K.pneumoniae*) probes. The results showed that the symbiotic genes were located together on one of plasmids, the symbiotic plasmid, with only one exception, in which *nod* and *nif* genes were not detected on plasmid in a strain containing a single plasmid although proved to be Nod$^+$ and Fix$^+$. The molecular weight of the symbiotic plasmid varied from 117 to 226 MD. *R.huakuii* strain 7653R is a Nod$^+$Fix$^+$ rhizobia symbiotically specific to *A.sinicus*. It harbours two large plasmids, pRh7a (115 MD) and pRh7b(210 MD). Strain 7653R-1 is a Nod$^-$ mutant derived from strain 7653R, which is devoid of the larger plasmid pRh7b (Zhou et al., 1987). In order to test the *sym*-plasmids response to early nodulation, experiment of root hairs infection was performed and the results are presented in Table 1. Root hairs on roots inoculated with strain 7653R show in average 58.32 percent of the elongated root hairs were deformed, and among them 24 "shepherd stick" were observed. Root hairs inoculated with strain 7653R-1 were not elongated, only 3.06 percent deformed and no "shepherd stick" observed. This indicated that the early nodulation genes *nod*DABC are located on the plasmid pRh7b, which is cured of in 7653R-1. The host-7653R-1 interaction stops at or before Hac stage.

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Hybridization with the probes of *nod*DABC and *nif*HDK showed that the tested genes were located on pRh7b.

Table 1. The test of root hairs infection

	Strain		
	CK	7653R	7653R-1
No.of tested plants	30	30	30
No. of RT hairs observed	1290	1562	1860
No. of RT hairs deformed	12	911	57
No. of " shepherd stick"	0	24	0
RT hairs deformed (%)	0.93	58.32	3.06

Behavior of exogenous *sym*-plasmid in *Rhizobium huakuii*

Some large rhizobia plasmids carrying symbiotic genes, such as pJB5JI, can be transferred between various *Rhizobium* species, but their behaviors are different in the new host strains, owing to the dissimilarities of genetic background (Beynon et al., 1980, Ruiz-Sainz et al., 1984, Wang et al., 1986). The aim of the experiment was to determine whether the *R.leguminosarum* host range plasmid pJB5JI could enable *R.huakuii* strain 7653R and its *sym*-plasmid-cured mutant 7653R-1 to form nodules on pea plants. The interaction of pJB5JI with the *sym*-plasmid of strain 7653R were studied. The *sym*-plasmid pJB5JI was transferred into *R.huakuii* strain 7653R and its *sym*-plasmid-cured mutant 7653R-1. Transconjugant 7653R-1 (pJB5JI) acquired tha ability to form ineffective nodules on pea plants (Zhang et al., 1992), whereas transconjugant 7653R (pJB5JI) did not. This indicatied that the indigenous *sym*-plasmid pRh7b restricted the functional expression of pJB5JI. Little is known about the mechanism of this. It is possible that the Nod factor produced by pJB5JI was modified by part of the *nod* genes on pRh7b, and could not be recognized by *Pisum sativum*. On the other hand,transconjugant 7653R (pJB5JI) showed higher nitrogenase activity on *A.sinicus* and higher dry weight (shoot) than those of the recipient strain 7653R (Zhang et al., 1995). The result is shown in the Table 2.

The exogenous plasmid pJB5JI in *R.huakuii* strains 7653R and 7653R-1 remained stable during successive transfer on culture media. But in part of the isolates from nodules formed by them, the pJB5JI was not visualized on gel by Eckhardt procedure. Southern hybridization using Tn5 and *nod* gene probes showed that these isolates still reserved, at least part of DNA of pJB5JI, which was probably integrated onto the chromosome.

Table 2. Nitrogen Fixation abilities of transconjugant

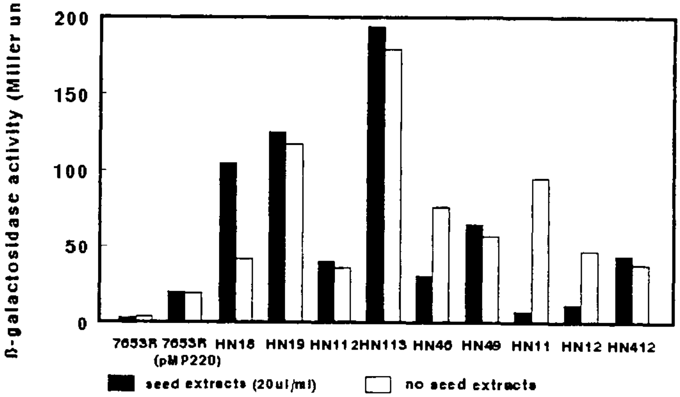
Inoculants	Acetylene reduced activity (μmol C_2H_4/g hour)	Shoot dry weight (g/plant)
7653R	15.8	0.1074
7653 (pJB5JI)	30.7[a]	0.1203
CK		0.0339

[a]: The difference compared to the parent strain 7653 is significant at level of $p=0.01$

Cloning and analysis of the regulation activity fragment of the *nod* genes in *R.huakuii* strain 7653R

It is known the *nod* promoter, NodD protein and flavonoid inducers are neccessary for the expression of *nod*ABC (Mulligan, Long, 1985; Rossen et al., 1985). In order to clone early nodulation genes *nod*ABC promoter, DNA fragments of the *sym*-plasmid (pRh7b) from the *R.huakuii* strain 7653R were cloned into the transcriptional fusion vector pMP220 (Spaink et al., 1987) to construct the *lacZ* fusions. The *lacZ* fusions so constructed were transferred into strain 7653R and blue colonies were selected on SM plates containing *A. sinicus* seed extracts, x-gal (100 mg/ml), Sm (100 mg/ml) and Tc (10 mg/ml). A number of the blue colonies were chosen to assay β-galactosidase activity with or without *A.sinicus* seed extracts by Miller Assay (Miller, 1972). Transconjugants (blue colonies) were respectively dissignated HN11, HN12. As shown in Figure 1, the endogenous β-galactosidase activity of *R.huakuii* strain 7653R was very low. The β-galactosidase activity of transconjugant HN18 was higher in presence of seed extracts as compared with its absence. The β-galactosidase activity of transconjugant HN11, HN12, HN46 were lower in presence of seed extracts than without seed extracts. With or without seed extracts, the β-galactosidase activities of transconjugants HN19, HN112, HN113 were of the same level. So, the result indicated that the transconjugant HN18 might have contained the *nod*ABC promoter, or the regulating activity fragment of the *nod* genes.

Fig.1 The ß-galactosidase activity of transconjuants (blue colonies)



Subsequently, the plasmid (pHN18) isolated from transconjugant HN18 and retransferred into *E. coli* DH5a for genetic analysis. Hybridization of plasmid pHN18 with *nod*DABC (from *R.leguminosarum*) probe showed that there was a 1.7kb homogenous band. The plasmid pHN18 was transferred into *sym*-plasmid-cured Nod$^-$ mutant strain 7653R-1. Transconjugants 7653R-1 (pHN18) retained the induction activity in presence of seed extracts, which signified that the expressioin of plasmid pHN18 was independent of the symbiotic plasmid (pRh7b). Three alternatives might comfirm the experimental results: 1) pHN18 contains *nod*D and *nod*ABC promoter from plasmid pRh7b. 2) pHN18 contains *nod* promoter only and *nod*D is located somewhere else, say on chromosome, or 3) pHN18 contains *nod*D and *nod* promoter, and there is another *nod*D somewhere else.

Preliminary identification of *R.huakuii nod* gene inducers

To determine which of the flavonoids are able to induce *nod-lacZ* fusion of *R.huakuii*, responses to 14 different compounds were tested. The results is given in Table 3. A betain trigonelline and a flavonoid luteolin did induce *nod-lacZ* fusion of HN18 strain. Trigonelline and *A.sinicus* seed extracts were used respectively to culture *R.huakuii* strain 7653R on SM liquid medium. The extracts of bacteria cultures were prepared by centrifugation, filtration and concentration. The cell free concentrates were applied to the roots of cell-free *A.sinicus* seedlings. The results showed that cell-free extracts prepared from cultures with trigonelline or with *A.sinicus* seed extracts were able to cause the elongation and deformation of the root hairs, which signified the presence of Nod factor produced by trigonelline induction in bacteria culture extracts.

Table 3. Screen for *nod-lacZ* inducibility by 14 compounds

Compounds (10 μm)	β-galactosidase activity (Miller units)	Compounds (10μm)	β-galactosidase activity (Miller units)
Apigenin	315	Naringin	248
Biochanin A	194	Quercetin	10
Chalcone	361	Rutin (non-flavonoid)	275
Flavanon	367	Trigonellin (non-flavonoid)	925
Formononetin	36	Umbelliferon	392
Genistein	90	Zeatin (non-flavonoid)	292
Luteolin	871	Seed extract	1154
Naringenin	277	O(CK)	382

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GENOMIC FOOTPRINTING ANALYSES OF THE COMPLEX *Bradyrhizobium japonicum fixRnifA* PROMOTER REGION.

BARRIOS H.[1], FISCHER H.-M.[2], HENNECKE H.[2] and MORETT E.[1]
[1]Instituto de Biotecnología, UNAM. AP 510-3, Cuernavaca, Mor. Mexico, and [2]Mikrobiologisches Institut, ETH. Zurich, CH-8092, Switzerland.

The *B. japonicum* NifA protein is the central regulator for nitrogen fixation gene expression. This protein is directly regulated by oxygen, such that it can only activate *nif* and *fix* gene expression during microaerobiosis or in root-nodule bacteroid state. NifA activates transcription by binding to enhancer-like elements, located around 120bp from the transcription start, and melting the closed promoter complex to the active open form. NifA is encoded in the *fixRnifA* operon. This operon is positively autoregulated in anaerobiosis and, unexpectedly, it is also expressed in aerobic conditions. Deletion and mutagenesis analyses have demonstrated that a region around -68 is required for the aerobic expression. This region is the binding site for an unknown protein. Here, we report that this pattern of expression is due to the presence of two overlapping promoters: fixRp1, which is of the -24/-12 class recognized by the RNA polymerase-σ^{54} (Eσ^{54}), and fixRp2, which shares homology with the -35 and -10 regions found in other putative *B. japonicum* housekeeping promoters, recognized by Eσ^{90}. Primer extension analyses showed that fixRp1 directs the synthesis of a transcript, denominated P1, that starts 12 nucleotides downstream of the -12 region. In addition to σ^{54}, P1 was dependent on NifA and low oxygen tension. Transcripts originating from fixRp2 started at two sites: one coincided with P1, while the most abundant, denominated P2, initiates just two nucleotides further downstream of P1. Expression from fixRp2 was dependent on the integrity of the upstream -68 promoter region but it was independent of σ^{54} and NifA. This promoter was expressed in aerobic and anaerobic conditions but was not expressed in bacteroids. Mutations in the -12 region for the σ^{54} promoter did not show any transcript because these mutations also disrupted the overlapping -10 region of the fixRp2 promoter. Conversely, mutations at the -24 region only affected the σ^{54}-dependent P1 transcript, having no effect on the expression of P2. In the absence of σ^{54} the anaerobic expression from the fixRp2 promoter was enhanced. suggesting that in the wild type strain the two RNA polymerase holoenzymes compete for the binding to the same promoter region.

We also showed by *in vivo* genomic DMS and $KMnO_4$ footprinting analyses that the *fixRnifA* promoter region is differentially protected and melted depending upon which RNA polymerase holoenzyme is bound. G residues at position -25, -24 and -13, the most conserved positions in σ^{54} promoters, were specifically protected in the wild type strain, but not in a strain devoid of σ^{54}. The region melted by Eσ^{90} was more extended than the open complex observed with Eσ^{54}. On the other hand, a G residue located at position -75 was specifically protected from methylation in the wild type promoter but not in a promoter that bears a A to C transversion at -68. Furthermore, two G residues, located at -100 and -121, were protected from methylation. These Gs form part of TGT motifs of UAS half-sites and are very likely binding sites for NifA.

EXPRESSION ANALYSIS OF HYDROGENASE STRUCTURAL GENES (*hupSL*) FROM *Rhizobium leguminosarum* bv viciae

BRITO, B.[1], A. TOFFANIN[1], D. FERNÁNDEZ[1], L. REY[1], J.M. PALACIOS[1], J. IMPERIAL[1,2], and T. RUIZ-ARGÜESO[1]
(1) Lab. Microbiología and (2) C.S.I.C; ETS Ingenieros Agrónomos, Univ. Politécnica de Madrid, 28040 Madrid, Spain

The genetic determinants for the H_2-uptake (*hup*) system of *Rhizobium leguminosarum* bv viciae UPM791 have been characterized in our laboratory. At least 18 genes are necessary for synthesis, assembly, and regulation of a functional uptake hydrogenase, responsible for recycling the H_2 generated in the nitrogenase reaction. The first 12 genes (*hupSLCDEFGHIJK hypA*) are only expressed in symbiosis with the legume host. Hydrogenase structural genes (*hupSL*) are transcribed from a -24/-12 promoter (P1). Deletion analysis identified an 85 bp region (positions -87 to -172) essential for activation. Within this region, an IHF-binding consensus sequence is present, and binding of *E. coli* IHF to it was demonstrated. Also present is a motif (ψUAS) reminiscent of known NifA-upstream activating sequences (UAS). We have followed three approaches to study the regulation of P1 expression: 1.- Study of the spatial/ temporal pattern of expression of P1 in pea nodules by *in situ* hybridization. We were able to show that *hupSL* are exactly co-expressed with *nif* genes, suggesting that they respond to similar environmental stimuli and activating proteins. 2.- Study of the effect of NifA on P1 activation. By using *hupL-lacZ* fusions in the heterologous *Klebsiella pneumoniae* host, NifA-dependent expression of P1 promoter was demonstrated. These results strongly suggest that, in *R. leguminosarum*, the expression of *hupSL* genes is under control of NifA, probably through binding to the identified NifA ψUAS. 3.- Search for regulatory genes homologous to *Bradyrhizobium japonicum hoxA, hupT,* and *hupUV* within the *hup* cluster. Sequence analysis of DNA upstream of *hupS* and downstream of *hupX* was carried out. A degenerate *hoxA* gene (ψ*hoxA*), encoding a polypeptide of 136 amino acid residues homologous to the N-terminal part of *hoxA*, was found downstream of *hupX*. Upstream of *hupS* no sequences homologous to *hupUV* were present. Instead, four ORFs, including a chemotaxis (*mcpA*-like) gene, were identified. Mutant analysis indicated that these ORFs are not involved in H_2 oxidation. 4.- Study of the legume host effect. For a given rhizobial strain, permissive legume hosts (peas) allow hydrogenase expression, whereas non-permissive hosts (lentils) do not. Immunological analysis showed that the level of HupSL proteins was lower in lentils than in peas, suggesting that the legume host effect is exerted at the level of *hupSL* gene transcription. The mechanism by which the plant controls *hup* gene expression is currently under study. Supported by CICYT (BIO93-0046) and DGICYT (PB91-0120).

DIFFERENT *RHIZOBIUM ETLI* PLASMIDS CONTRIBUTE TO THE ESTABLISHMENT OF AN EFFECTIVE SYMBIOSIS.

BROM, S., GALVAN, L., GIRARD, L., GARCIA DE LOS SANTOS, A., PALACIOS, R., AND ROMERO, D.
Depto. de Genetica Molecular, Centro de Investigacion sobre
Fijacion de Nitrogeno. UNAM. A.P. 565-A, Cuernavaca, Morelos,
Mexico

Rhizobium etli strain CFN42 contains six plasmids (pa to pf) whose sizes range between180 and 600 kb. The analysis of derivatives cured (or deleted for pe) of each individual plasmid, allowed us to display the participation of these plasmids in different symbiotic and saprophytic functions (Brom et al.,1992), including: nodulation (pb and pd), competitivity (pb, pc, pe and pf), and growth on minimal (pa,pb,pc,pd,pe and pf) and rich (pc, pe and pf) media.
Recently, we have determined the nitrogen fixation ability of these strains, under greenhouse conditions. The results show a requirement of plasmids c, e and f to reach wild type nitrogen fixation levels. Derivatives containing a variety of plasmid combinations were constructed. The analysis of their symbiotic (nitrogen fixation and competitivity for nodulation) and free-living (growth rates on minimal and rich media), indicate that among the cellular functions that require the expression of plasmid-encoded genetic information, some depend on genetic information ascribed to a particular plasmid, while the wild type level of other functions depend on the cooperative interaction of genetic information distributed among different replicons.

Brom S et al (1992) J. Bacteriol. 174, 5183-5189

MOLECULAR GENETIC ANALYSIS OF *BRADYRHIZOBIUM JAPONICUM GLT* LOCUS

CHATUEV B.M., SADYKHOV M.R., IVASHINA T.V.,
KSENZENKO V.N.
Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow Region, 142292, Russia

Glutamate synthase (GOGAT) is one of the key enzyme responsible for ammonium assimilation in different bacteria involving Rhizobiaceae. GOGAT activity is necessary for effective simbiotic assosiation between *B.japonicum*, A.sesbania and their hosts (O'Gara et al., 1984; Chatuev et al., 1985; Hilgert et al., 1987). However, the precise functional role of the glt genes (GOGAT) in nitrogen metabolism and nitrogen fixation is still unclear.

The cosmid clone pBT53*glt* encoding GOGAT was selected from the pLAFR genomic bank of *B.japonicum* 110 by complementation of *B.japonicum* Asm strains (Chatuev et al., 1985). The cloned *glt* region was able to restore Glt$^+$Asm$^+$Fix$^+$ phenotype of *B.japonicum* Asm104 as well as to complement glt mutation in R.meliloti, A.sesbania and *E.coli* with the rehabilitating of GOGAT activity in this strain.

The 40 mutant pBT53*glt*::*Tn*5 cosmids with insertions of *Tn*5 within the four different *EcoR*I fragments of cloned DNA were isolated. Transconjugants of *E.coli* PA340, *R.meliloti* AK330 (Kondorosi et al., 1977) and *B.japonicum* Asm104 strains harboring pBT53glt ::*Tn*5 plasmids showed the similar GltAsm phenotype. The combined genetic and physical maps of cloned 21 kb DNA region were constructed and the positions of the *Tn*5 insertions within cloning fragment were identified by complementation and restriction analysis of the pBT53*glt*::*Tn*5 plasmids and their DNA's, respectively. The positions of the six *glt*::*Tn*5 insertions led to complementation inability of the *glt* locus were mapped by sequencing of the transposon-genome DNA junctions. The data on partial sequencing of the four *EcoR*I DNA fragments of cloned region allowed to identify the *gltB* gene of *B.japonicum*110. Three *glt*::*kan* constructs involving pSUP101*gltB*::*kan* plasmid carrying the *kan*-cassette cloned within the *gltB* gene were obtained. Isolation of the *B.japonicum gltB*::*kan*, as well as another insertional *glt*::*kan* mutants are in progress.

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HEALING OF A COMPETITIVE DEFECT IN A STRAIN OF *Rhizobium etli* THROUGH SPECIFIC COMPLEMENTATION OR BY EPISTATIC INTERACTIONS.

CORVERA, A., EPARVIER, A. AND ROMERO, D.
Dept. of Molecular Genetics, CIFN-UNAM, Ap. Postal 565-A,
Cuernavaca, Morelos, Mexico

The symbiotic properties of a strain carrying a rearrangement in the p*Sym* of *Rhizobium etli* CFN42 were studied. This rearrangement was generated by recombination between a 170 Kb *nod-nif* region of the p*Sym* and an endogenous plasmid (pa), thus leading to the loss of a 220 Kb region from the p*Sym*. The strain (CFNX250) harboring this rearranged p*Sym* (pGM1) shows an increase in symbiotic effectiveness as compared to the parental strain (Romero et.al., this congress), but also a strong decrease in nodulation competitiveness (i.e. CFNX250 coinoculated with CFN42 in an 1:1 ratio occupies only 10 % of the nodules). This phenotype has been complemented,albeit partially, with defined sectors of the p*Sym* , leading us to the identification of a new p*Sym* locus involved in the competitiveness of *R. etli* CFN42. This locus is located in a sector of the p*Sym* previously thought to be cryptic.

We have found a second way to correct the loss of competitivity in a pGM1-bearing strain. A spontanous tandem duplication of a 120 kb region in pGM1 completely restores the competitive ability of the strain. Coinoculation assays between CFN42 and CFNX253 (harboring pGM1 with a tandem duplication) in a 1:1 ratio result in equal nodule occupancy for both strains. Our hypothesis is that determinants located in the 120 kb region cooperate with the newly-described locus to achieve wild-type competitivity. The phenotypic effects of the loss of the new locus can be compensated by tandem duplication of a different region, thus constituting an example of epistatic interactions for nodulation competitiveness.

IDENTIFICATION AND FUNCTIONAL ANALYSIS OF THE *RHIZOBIUM LEGUMINOSARUM* BIOVAR *PHASEOLI* CNPAF512 *ARCABC* GENES.

D'HOOGHE, I., DE WILDE, P., VERRETH, C., VANDERLEYDEN, J.
F.A. Janssens Laboratory of Genetics, W. de Croylaan 432, B-3001
Heverlee, Belgium

Sequence analysis upstream the *Rhizobium leguminosarum* bv. *phaseoli fixLJ* genes revealed the presence of three open reading frames. These open reading frames showed strong homology with the *arcABC* genes of *Pseudomonas aeruginosa*. The *P. aeruginosa arcABC* genes encode the enzymes of the arginine deiminase pathway (Baur *et al.*, 1989). This pathway is essential for the generation of ATP from arginine in oxygen-limiting conditions (Gamper *et al.*, 1991). Arginine is converted into ornithine and carbamoylphosphate, which serves to generate ATP from ADP. No homology has been found so far with the fourth gene of the *P. aeruginosa arc* operon, the *arcD* gene which encodes a membrane-bound arginine-ornithine antiporter. The corresponding amino acid sequences were determined and aligned with the amino acid sequences of the *P. aeruginosa* ArcA, ArcB and ArcC proteins. The *R. leguminosarum* bv. *phaseoli* ArcA, ArcB and ArcC proteins were highly homologous with their counterparts in *P. aeruginosa*: 62% identical amino acids for the ArcA proteins, 72% for the ArcB proteins and 65% for the ArcC proteins.

The *arcABC* genes have not been described previously in *Rhizobium* species. Therefore we studied the presence of *arc* genes in different *Rhizobium* species by hybridization on genomic DNA of these bacteria with a fragment containing the *R. leguminosarum* bv. *phaseoli arcAB* genes. Surprisingly hybridization signals were only observed in the *R. leguminosarum* bv. *phaseoli* strains, but not in the other tested strains.

To study the function of the *arcABC* genes in *R. leguminosarum* bv. *phaseoli* and their possible role in symbiotic nitrogen fixation, *R. leguminosarum* bv. *phaseoli arcABC* mutants were constructed by insertion mutagenesis. The *R. leguminosarum* bv. *phaseoli arcABC* mutants were still able to fix nitrogen. However the two *arcABC* mutants showed a lower acetylene-reduction activity than the wild-type. No significant differences were found for the nodule-dry-weight, plant-dry-weight and number of nodules.

Baur *et al.*, Eur. J. Biochem., **179**: 53-60.
Gamper *et al.*, J. Bacteriol., **173**: 4742-4750.

THE NODULATION DEFECTIVE PHENOTYPE OF A TN5-INDUCED PURINE AUXOTROPH OF *RHIZOBIUM* STRAIN NGR234 ON SIRATRO IS ALTERED BY THE PRESENCE OF 5AMINOIMIDAZOLE-4-CARBOXIMIDE-RIBOSIDE

DJORDJEVIC, S.P. [1], WEINMANN, J.J., REDMOND, J.W.,
DJORDJEVIC, M.A., ROLFE, B.G.
Centre for Genetic Research (Plant Microbe Interaction Group), Research School of Biological Sciences, The Australian National University, Canberra, ACT 0200, Australia.
(1) NSW Agriculture, Elizabeth Macarthur Agricultural Institute, Microbiology & Immunology Section, PMB 8, Camden, NSW, Australia.

Transposon Tn*5*-induced purine mutants (ANU2861 and ANU2866) of the broad-host range *Rhizobium* strain NGR234 are defective for nodule formation on a wide variety of legumes and the non-legume *Parasponia.* In strain ANU2861, complementation studies and DNA sequence analysis of regions, on either side of the insertion of Tn*5*, revealed two open reading frames with strong sequence homology to the *purM* and *purN* genes of *Escherichia coli.* Strain ANU2861 contained a single copy of Tn*5* inserted into the carboxy-terminal portion of the *purM* gene and these two purine genes are likely to form part of an operon in strain NGR234. Mutant ANU2866 was complemented by a cosmid containing the purine biosynthetic genes *purY, purQ* and *purL.* Genes *purQL* code for enzymes required before AICAR synthesis. The addition of 0.1 mM 5-aminoimidazole-4-carboxamide riboside (AICA riboside) to MM agarose medium enabled both mutants ANU2861 and ANU2866 to form colonies. On *Macroptilium atropurpureum* (siratro) ANU2861 rapidly induces a necrotic response at the site of infection (Djordjevic *et al.*, 1988, However, when the plant growth medium was supplemented with 0.1 mM AICA-riboside, both strains ANU2861 and ANU2866 induced the formation of small nodule-like structures on the roots of siratro which (1) do not contain bacteria, (2) lack infection threads, and (3) possess centrally-located vascular bundles in contrast to the peripheral location in functional nodules induced by a parent strain ANU280. When mutant ANU2861 was spot inoculated onto the roots of siratro, or coinoculated onto the roots with the ExoY mutant ANU2811, a thick and short root (Tsr) phenotype was induced. Siratro inoculated with strain ANU2861 grown in the presence of 0.1 mM ACICA riboside showed a greatly reduced Tsr phenotype. Siratro roots inoculated with strain ANU280 do not induce a Tsr-like phenotype. Polyacrylamide gel electrophoresis of the lipopolysaccharide (LPS) isolated from strain ANU2861 showed a modified LPS profile when compared with the wild-type strain ANU280. Growth of ANU2861 in medium supplemented with AICA-riboside corrected this modified LPS profile. The exopolysaccharides (EPS) of strains ANU2861 and ANU2866 are indistinguishable from those of parent strain ANU280. Thus the synthesis of the activated sugar precursors can not be impaired. The deficiency in the LPS synthesis of ANU2861 is likely to be due to a defect in the synthesis of a particular constituent monosaccharide. In contrast, the purine mutant ANU2866, which has a Nod^- phenotype, has no apparent alteration to its LPS synthesis. We conclude that a particular intermediate in the purine biosynthetic pathway, in addition to the CLOS molecules, is essential to the early stages of nodule development on at least some plants.

Djordjevic et al (1988) J. Bacteriol. 170, 1848.

ANTHOCYANIN-INDUCED O-ANTIGEN MODIFICATION DIMINISHED IN NODULATION-DEFICIENT *RHIZOBIUM* MUTANT

DUELLI D.M. (1), HONG T. (1), BREWIN N.J. (2), NOEL K.D. (1).
(1) Department of Biology, Marquette University, Milwaukee,
WI 53233, USA
(2) John Innes Institute, Norwich NR4 7UH, UK

Black bean (*Phaseolus vulgaris*) seed exudate induces an epitope change in *R.etli* CE3 O-Antigen (O-Ag). White bean seed exudate does not induce the O-Ag modification. The exudate-induced effect does not require known *nod* genes and is at least partially independent of the established *nod*-induction pathways. However, release of O-Ag modifying activity from black beans coincides temporally with exudation rates for *nod*-inducer release in black seeds. Preliminary isolation of O-Ag modifying agents in black bean seeds shows that at least four O-Ag modifying HPLC-fractions are rich in different anthocyanins. One commercially available anthocyanin, delphinidin, induces the O-Ag modification at 100 µM. These data suggest that the O-Ag modification accompanies *nod*-induction and is controlled by some of the same plant signals or ones that are simultaneously exuded with *nod*-inducers. A *R.etli* mutant strain, CE374, elicits smaller, more disperse and 80% fewer nodules on 15-day-old black bean roots than wild type parent CE3. The O-Ag of CE374 is modified only at higher exudate concentrations. These data suggest that nodulation proficiency depends on O-Ag modification.

RHIZOBIUM MELILOTI MUTANTS DEFECTIVE IN HIGH- AFFINITY IRON ACQUISITION

FABIANO E., GILL, P., BAGNASCO, P., NOYA, F., DE LA FUENTE, L. AND ARIAS, A.
Biochemistry Dept. IIBCE. Ave. Italia 3318.Montevideo
11600.Uruguay.

Environmental iron is present in an oxidized and polymerized state which is extremely insoluble at biological pHs. Microbes typically respond by producing extracellular low molecular weight chelator, *i.e.* siderophores, and membrane transport systems for specific uptake of their ferric complexes. It was previously shown that the iron requirement is higher for the symbiosis than for the free-living form of rhizobia, nonetheless limited information is available about the iron acquisition systems of rhizobia.

The occurrence of high affinity iron transport systems is widespread among native Uruguayan rhizobia (Fabiano et al.,1994). An effective native strain (Rm 242) isolated from nodules of alfalfa, was shown to produce siderophore activity that was functionally distinct from that of strain Rm 1021.

The aim of the present work is to characterize the iron transport system present in the native strain Rm 242.

Tn5-mob induced mutagenesis of Rm 242 was done using standard methods. Three general classes of mutants were found by using the CAS assay: I) those that produced not detectable or functional siderophore, ii) those that overproduce a functional siderophore but which were unable to utilize it, iii) one that overproduce a functional siderophore in both iron limiting and in iron sufficient conditions.

Our results extend previous finding using different strain of *R.meliloti* (Gill and Neilands,1989), showing that rhizobia presents a siderophore mediated iron acquisition system. This system is repressed in iron sufficient media. According the properties of the *Tn5-mob* induced mutants, this system appears to be complex, involving different components.

The results obtained in plant assays suggest that the high-affinity iron acquisition system expressed by the free-living form is not essential for the nitrogen-fixation although it can affect the early events of nodulation.

Siderophore mediated competition is a widespread phenomena among soil microorganisms. The mutants obtained in this work will be a useful tool to study the iron uptake role in the competition between rhizobia and other soil microorganisms.

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INVESTIGATION OF THE ILVC GENE IN SPECIES OF *RHIZOBIUM* AND *BRADYRHIZOBIUM*

GRASSO, D.H., GALINDO-BLAHA, C.A., RICCILLO, P., PAGANO, M. AND AGUILAR O.M.
Instituto de Bioquinica y Biologia Molecular, Facultad de Ciencias Exactas, Universidad Nacional de la Plata, 1900-La Plata, Argentina

We had demonstrated the requirement of a functional *ilvC* gene of *R.meliloti* for the nodulation of alfalfa (Aguilar, Grasso, 1991). IlvC mutants of *R. meliloti* are auxotrophs for ile and val and are Nod$^-$ on alfalfa. We have initiated studies to address the question whether mutations in the *ilvC* gene of other species of *Rhizobium* and *Bradyrhizobium* have any effect on the symbiotic interaction with the respective host legume. After careful inspection of the *ilvC* sequence of *R.meliloti*, *E.coli* and *S.cerevisae*, we designed oligonucleotides to use them in the PCR reaction. DNAs from *R.etli*, *R.trifolii* and *B.japonicum* yielded amplification products of about 500 bp. The ilvC-PCR amplification product from *R.etli* CE3 and from *B.japonicum* 110 were purified and cloned in vector plasmids that do not replicate in a rhizobial background ("suicide vector"). By using these recombinant plasmids, insertional mutants were isolated in which the wild type *ilvC* gene was interupted by the plasmid as consecuence of a single event of homologous recombination. The mutations were confirmed by Southern analysis. These mutants of *R.etli* and of *B.japonicum* were unable to grow on minimal medium GTS unless supplemented with amino acids isoleucine and valine. This phenotype indicates that the amplified region had integrated into a functional *ilvC* sequence. These *ilvC* mutants will be used in plant inoculation experiments to examine the significance of the *ilvC* in the symbiosis with the respective host.
In addition, the expression of the *R.meliloti* ilvC gene was studied by using 5'-ilvC-lacZ gene fusions. The *ilvC-lac Z* fusions were assayed in *R.meliloti* in both the *IlvC* and the wild type backgrounds. It was found a constitutive level of expression (about 150-200 Miller units) that increases about 5 folds during the late log phase of growth. The effect of the presence of amino acids isoleucine and valine was also examined and found a similar level of expression. It was also found that the level of activity was higher in the IlvC mutant than in the wild type *R.meliloti* 1021. This suggests that in the *ilvC* background, the substrates of the IlvC protein (isomeroreductase) may accumulate in the mutant cells and play a cooperative role in the positive activation of the *ilvC* expression.

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THE *Rhizobium leguminosarum* bv viciae *hypBFCDE* OPERON IS TRANSCRIBED FROM A FixK/FnrN DEPENDENT PROMOTER LOCATED WITHIN THE *hypA* GENE

HERNANDO, Y.[1], L. REY[1], D. FERNÁNDEZ[1],J.M. PALACIOS[1], J. IMPERIAL[1,2], and T. RUIZ-ARGÜESO[1]
(1) Lab. Microbiología and (2) C.S.I.C; ETS Ingenieros Agrónomos, Univ. Politécnica de Madrid, 28040 Madrid, Spain

Pea (*Pisum sativum* L.) bacteroids produced by *Rhizobium leguminosarum* bv viciae UPM791 synthesize a membrane-bound, (NiFe) hydrogenase which oxidizes the H_2 arising from nitrogen fixation in root nodules. The synthesis of the active enzyme requires the proteins from the structural genes (*hupSL*) and an array of accessory proteins from at least 16 additional genes including the gene cluster *hypA,B,F,C,D,E*, likely involved in nickel metabolism. Unlike the *hupSL* genes, which are only expressed in symbiosis, the *hypBFCDE* operon is also activated in vegetative cells in response to low pO_2 in the culture media. In microaerobic cells, transcription of the *hypBFCDE* operon occurs from two promoters , P5a and P5b, located 100 bp and 190 bp upstream of the ATG start codon of *hypB*, respectively, within the coding sequence of *hypA*. The P5b transcription start site is preceded by an Fnr box (anaerobox), 5' TTGAgccatgTCAA 3', centered at position -40. The P5a transcription start site is preceded by a σ^{54}-binding sequence at position -13. Promoter deletion experiments revealed that a 248 bp DNA region containing the anaerobox is essential for microaerobic expression of both promoters. In pea bacteroids only transcription from promoter P5b was detected. Expression of both P5a and P5b promoters in the heterologous *R. meliloti* bacterial host is dependent on the presence of an active *fixK* gene. In *R. leguminosarum* UPM791, an *fnrN* gene was identified that, when provided in a plasmid base, is able to replace *R. meliloti fixK* gene for activation of both *fix* genes and *hypBFCDE* operon, probably by binding to FixK recognition sequences upstream of *fix* genes and to the anaerobox in P5b. Microaerobic expression of a *hypB::lacZ* fusion in a genomic *R. leguminosarum* UPM791 *fnrN*$^-$ mutant was reduced by 60% as compared to the wild-type strain. However, symbiotic expression of *hyp* operon and hydrogenase activity of pea bacteroids were not affected in the *fnrN*$^-$ mutant . The presence of a second gene of the *fixK/fnr* type, functionally equivalent to *fnrN*, in *R. leguminosarum* UPM791 was inferred from these results, and was also supported by Southern hybridization assays using an *fnrN* internal DNA probe. This gene was isolated, partially sequenced, and shown to be a second *fnrN* gene (*fnrN*$_2$). $FnrN_2^-$ and double $FnrN_1^-$ $FnrN_2^-$ mutants are presently being characterized to investigate the role of each gene in *fix* and *hyp* gene activation in *R. leguminosarum*. Supported by CICYT (BIO93-0046) and DGICYT (PB91-0120).

MOLECULAR GENETIC ANALYSIS OF *RHIZOBIUM LEGUMINOSARUM* BV. *VICEAE* VF39 *PSS* GENES

IVASHINA T.V., SADYKHOV M.R., CHATUEV B.M.,
DMITRIEV V.V., SUZINA N.E., DUDA V.I., KSENZENKO V.N.
Institute of Biochemistry and Physiology of Microorganisms,
Russian Academy of Sciences, Pushchino, Moscow Region, 142292,
Russia

Two chromosomal regions, involved in exopolysaccharide production and nodulation of plants of pea cross-inoculation group (*Pisum sativum*, *Vicia sativa* and *V.faba*) have been identified in *Rhizobium leguminosarum* bv. *viceae* VF39. Nucleotide sequence analysis of a 3045 bp *Eco*RV-*Sac*I fragment revealed the presence of two ORFs, designated as *pss*1 and *pss*4 genes. Transcription start point mapping data (Ivashina et al., 1994) and results of analysis of *pss*4 mutants, carrying mutations of the central part of the gene and its 5' end, suggest the existance of two forms of Pss4 (probably glucosyl transferases) The larger one (263 aa) besides the catalic function, can act as an adhesion cell molecule. The product of the *pss*1 gene, localized upstream from the *pss*4, may be attributed to the family of diverse proteins homologous to inositol monophosphatase (Neuwald et al. 1991). The highest level of homology (40% of identity) was observed with AmtA/CysQ from *E.coli* responsible for cysteine biosynthesis (Neuwald et al. 1992) and (or) ammonium transport (Fabiny et al. 1991). All the mutations obtained by the insertion of the kan-cassette into different parts of the *pss*1 failed to complement *E.coli cysQ* but only some of them led to the alteration of symbiotic properties in VF39 background (Nod$^+$Fix$^-$). No differences in the ultrastructure of nodules induced by the *pss1* mutants and the wild type strain were found. However, alterations in the osmiophility of cell wall outer surface of free-living mutants were detected. Nucleotide sequence of a 3640 bp *Eco*RI-*Mlu*I fragment revealed four ORFs incoding proteins with predicted molecular weights of 54.6, 37.1, 15.8 and 16.0 kDa, respectively. No significant homology of ORF1 was found in the data bank except for the presence of short region with similarity to *R.meliloti* ExoW, ExoO as well as to NodC from different *Rhizobium* species. The deduced aa sequence of ORF2 showed overall homology to *R.meliloti*, which was proposed to be glucosyl transferase, requierd for synthesis of the octasacchride subunits of succinoglycan (Reuber, Walker 1993). Two others ORFs displayed no homology to any known proteins and can be attributed to new genes essential for EPS production. Tn5 insertions in these ORFs completely abolished nodulation of *V.faba* and resulted in EPS- phenotype.

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IDENTIFICATION OF SOIL BACTERIA EXPRESSING A SYMBIOTIC PLASMID FROM *RHIZOBIUM LEGUMINOSARUM* BIOVAR *TRIFOLII*

JARVIS B.D.W., SIVAKUMARAN S., LOCKHART P.J.
Department of Microbiology and Genetics, Massey University, Palmerston North, New Zealand

Biotic interactions between *Rhizobium* seed inoculants and other soil bacteria have not received much attention but they play a critical role in determining inoculant competitiveness and survival in field. This study concerned the identification of soil bacteria which were unable to nodulate white colver (*Trifolium repens*) cv. Grasslands Huia but could accept a transconjugant symbiotic plasmid from *Rhizobium leguminosarum* bv. *trifolii* strain Rtr514a and subsequently establish symbiosis with clover. This work follows earlier studies in which we have shown the expression by unidentified soil bacteria of nodulation genes from *R. leg.* bv. *trifolii* (Jarvis *et al.*, 1989) and the competitiveness of the transconjugants in relation to inoculant strains (Rao *et al.*, 1994). Earlier studies (Soberon-Chavez, Najera, 1989; Segovia *et al.*, 1991) concerned non-symbiotic bean rhizobia. We hypothesised that the nature of the non-symbiotic bacterial population capable of expressing a *Sym* plasmid was unknown and could include other bacteria in addition to those recognisable as rhizobia. We isolated 100 Gram negative soil bacteria which could not nodulate white clover and transferred symbiotic genes to them by conjugation with an *Escherichia coli* strain PN200 containing a co-intergrate plasmid pPN1 comprising R68.45 and pRtr514a from *R. leg.* bv. *trifolii* strain Rtr514a. Ten strains gave transconjugants which nodulated white clover. These strains contained pPN1 and examination by light and electron microscopy showed bacteriod filled plant cells in the nodules. Nodule isolates identical with the recipient bacteria and strains from earlier work were characterised by ribosomal fingerprinting, partial 16S rRNA sequence analysis using Y1 and Y2 primers (Young *et al.*, 1991) DNA-DNA hybridisation (Ramadass *et al.*, 1992) and analysis of total fatty acids (Jarvis, Tighe, 1994) and identified as *R. etli* (1 strain); *R. leguminosarum* (6); *R. loti* (2); *R. meliloti* (1); *R. tropici* (1) and *Rhizobium* sp. (4). It was concluded that pasture soils contained and unexpected variety of non-nodulating rhizobia and that the *Sym* plasmid pRtr514a was capable of expression in them but the hypothesis that other Gram negative soil bacteria might express a *Sym* plasmid from *R. leg.* bv. *trifolii* was not supported.

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SEARCH IN THE NATURAL POPULATIONS AND GENERATION BY THE PLASMID TRANSFER OF *RHIZOBIUM LEGUMINOSARUM* BV.*VICEAE* STRAINS, WHICH FORM HIGHLY-EFFECTIVE SYMBIOSIS WITH HAIRY VETCH (*VICIA VILLOSA* ROTH)

KURCHAK, O.N., PROVOROV, N.A., SIMAROV, B.V.
Research Institute for Agricultural Microbiology, Sh.Podbelsky 3, St.Petersburg - Pushkin 8, 189620, Russia

Hairy vetch (*Vicia villosa* Roth), one of the most important winter forage crops, is a macrosymbiont of *Rhizobium leguminosarum* bv.*viceae*.

From soils and nodules of 9 wild-growing and cultivated *Vicia* L. species collected at two geographically separated areas (Ukraine and Central Asia) we isolated 165 *R.l.*bv.*viceae* strains, which were tested for activity of symbiosis with hairy vetch in aseptic tube tests. We found that percentage of active strains from these two regions was nearly the same. Percentage of active strains, isolated from virgin soils of mountain regions was much higher than from cultivated soils of flat regions of Ukraine (80% and 35%, respectively). Plasmid profiles of 46 wild-type strains from Ukraine and 25 - from Central Asia showed that these two rhizobia populations differed in mean plasmid number per isolate (3 and 4, respectively).

In each of three most effective strains (exceeded commercial *R.l.*bv.*viceae* strain for ability to increase shoot mass by 14-22%, N accumulation by 28-33% in pot and field trials) three plasmids were found, the smallest of which were proved to be *Sym*-plasmids (p*Sym*) by means of transfer into *Agrobacterium* and *Nod⁻* *R.meliloti* strain CXM1-46.

To elucidate the role of p*Sym* in determination of symbiotic effectiveness, we introduced p*Sym* 1-32::Tn5-mob in *R.l.*bv.*viceae* strain Y57 (non-effective) and Y14 (low effective with *V.villosa*).In one case, we obtained transconjugants which caused significant increase in plant mass, N accumulation and acetylene-reduction as compared to recipient strain Y57. When we introduced p*Sym* 1-32::Tn5-mob in *R.l.*bv.*viceae* Y14, agarose gel electrophoresis revealed the presense of donor p*Sym* in all 14 analysed transconjugants. When we tested these transconjugants in sterile tube test (on *V.villosa* plants), some of them increased significantly plant mass (by 48-52%), N accumulation (by 51-59%) and nitrogenase activity (by 37-111%) as compared with recipient strain.

Hence, the mobilized plasmids are suggested to carry genes, necessary for the effective symbiosis with vetch. It can be also concluded that the symbiosis potential of a recipient strain can be improved in case if it is inferior to the donor. This approach we can use for construction of commercial strains, which combine high effectiveness with other practically important properties, such as specificity, competitiveness, stability, etc.

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LOCALIZATION OF *nodHPQ* GENES IN *Rhizobium tropici* CIAT899.

MARROQUÍ S. (1), MANYANI H. (1), FOLCH J. L. (2) AND MEGÍAS M. (1)
(1) Department of Microbiology and Parasitology, University of Seville, 41012 Seville, Spain.
(2) Department of Plant Molecular Biology, Institute of Biotechnology, U.N.A.M., Cuernavaca, Morelos 62271, Mexico.

The symbiosis established between rhizobia and leguminous plants is a host-specific process. One of the most important determinants of host specificity is the production of lipo-oligosaccharidic signal molecules by the bacterium in response to flavonoid compounds exuded by the host plant. The structure of these lipo-chitin oligosaccharides (LCOs) consists of an N-acetylglucosamine backbone of 3 to 5 units to which an acyl chain and other different specific substituents are attached by the enzymatic activities of the proteins encoded by some nodulation (*nod*) genes. In the case of the broad host range strain *R. tropici* CIAT899, two major families of LCOs have been found: the non-sulphated ones and the sulphated ones, the latter group carrying a sulphate moiety at position 6 of the molecule's reducing terminal sugar. This sulphate group is synthesized and attached to the LCO by the intervention of the *nodPQH* gene products. We have localized and sequenced this set of genes in the symbiotic plasmid of *R. tropici* CIAT899 and found that all three exhibit a very high homology when compared to the corresponding genes of *R. meliloti* (ranging from 67.755 to 76.777% identity at protein level). *nodPQ* are also very homologous to their *Azospirillum brasilense* counterparts and to the *Escherichia coli cysDNC* operon encoding ATP sulphurilase and APS kinase activities. We have also found the one-base overlapping motif TGATG between the stop codon of *nodP* and the initiation codon of *nodQ*, suggesting a translational unit organization as described for *R. meliloti nodPQ* genes. *nodH*, in contrast to its *R. meliloti* homologue, is located upstream of the *nodPQ* genes, thus suggesting that the three genes might form a translational unit. In addition, we have constructed mutants in *nodH* and *nodQ* genes and tested both mutant strains on *Phaseolus* and *Leucaena*, two non-related host plants of *R. tropici*. The results obtained indicate that the sulphation of LCOs probably plays a crucial role in the nodulation efficiency of *Leucaena* plants by *R. tropici* CIAT899.

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CHARACTERIZATION OF A PLEIOTROPIC MUTANT OF *AZORHIZOBIUM CAULINODANS* IMPAIRED IN AMMONIA ASSIMILATION AND IN NITROGEN FIXATION.

MICHEL-REYDELLET N., DESNOUES N., ELMERICH C., KAMINSKI P.A.
Unité de Physiologie Cellulaire, Département des Biotechnologies, Institut Pasteur, 75724 Paris Cedex 15 France.

Regulation of nitrogen fixation genes has been extensively studied in *Azorhizobium caulinodans*. The transcription of *nifH* requires RpoN and NifA, as in other diazotrophs from proteobacteria. *nifA* expression is regulated by oxygen through FixLJK, and its regulation by ammonia is only partially mediated through NtrBC and NtrYX.

Here, we report the characterization of the strain 5795, a Nod$^+$, Nif$^-$, Fix$^-$ mutant of *A. caulinodans* obtained after EMS mutagenesis. This strain displays commun features with a NtrC mutant:

- it is unable to grow on several nitrogen sources such as nitrate, histidine, arginine,
- *nifA* expression is partially affected (70% compared to the wild type) while *nifH* is not transcribed.

However, the mutant is more pleiotropic than *ntrC* since it is auxotroph for glutamine. This Gln$^-$ phenotype is correlated with a 90% decrease of the glutamine synthetase level, which does not result of a mutation in *glnA*. Indeed a constitutive *glnA* gene does not restore the wild type phenotype. The introduction of constitutive *nifA* or *rpoN* genes does not lead to a recovery of the nitrogen fixation suggesting that these genes are not affected by the mutation.

Prototrophy and nitrogen fixation ability can be restored in 5795, by a fragment of 2.5 kb which shares no similarity with any other regulatory genes previously characterized in *A. caulinodans*. The nucleotide sequencing of this fragment revealed an ORF whose translation product shares similarity with transcriptional activators of the LysR family. This ORF was designated *nac*-like on the basis of the high degree of identity between the gene product and Nac of *Klebsiella aerogenes*. As a current working hypothesis it is proposed that Nac-like would be one of the first elements of the signal transduction pathway that controls nitrogen metabolism.

EXPRESSION OF THE *RHIZOBIUM LEGUMINOSARUM* BV.*VICIAE HUP* SYSTEM IN HETEROLOGOUS RHIZOBIAL GENETIC BACKGROUNDS.

MONZA J.(1), BRITO B.(2), FERNANDEZ C.(2), CABRERA E.(2), CADAHIA E.(2), PALACIOS J.M.(2), IMPERIAL J.(2,3), RUIZ-ARGUESO T.(2).
(1) Dept. Bioquimica, Ftad. Agronomia, Universidad da la Republica, Montevideo, Uruguay.
(2) Lab.Microbiologia and (3) C.S.I.C., ETS Ingenieros Agronomos, Univ. Politecnica, 28040 Madrid, Spain.

The capacity to recycle the H_2 arising from nitrogen fixing system in legume nodules has been shown in only a reduced number of rhizobial strains. The genetic determinants for the H_2-uptake (*hup*) system have been isolated and characterised from *R.leguminosarum* bv. *viciae* and *Bradyrhizobium japonicum*. In order to extend this capacity to rhizobia nodulating agriculturally important crops, the expression of the hup system was investigated in heterologous bacterial hosts. To this aim, cosmid pAL618, containing the hup gene cluster (*hupSLCDEFGHIJKhypABFCDEhupX*) from *R.leg.*bv.*viciae* UPM791 (Hidalgo et al., 1990, 1992; Rey et al., 1992, 1993) was transferred into *hup*$^-$ strains of *R.loti, R.elti, R.leguminosarum* and *R.meliloti*, and the resulting transconjugants were tested for hydrogenase activity in free-living conditions and in symbiosis with birdsfoot, beans, peas and alfalfa, respectively. No hydrogenase activity associated to cosmid pAL618 was detected in microaerobically grown cells of any of the bacterial hosts. This lack of activity correlated well with the lack of expression of hydrogenase structural genes (*hupS::lacZ* fusion), althugh in all cases expression of *hyp* genes (*hypB::lacZ* fusion) was observed. In contrast, expression of hydrogenase activity was detected in bacteroids from all the strains tested, and the activity levels were found to be strain- and species-depent. A requirement of nickel supplementation for optimum hydrogenase activity was shown in the *R.leg.*/pea, *R.loti*/birdsfoot and *R.meliloti*/alfalfa symbioses. This nickel effect was exerted at the level of processing of hydrogenase structural proteins (Brito et al.,1994), and indicates a deficiency on the nickel provision to the bacteroid in these symbiosis. The nickel limitation may not occur in the *R.elti*/bean symbioses, where nickel addition has no effect on either hydrogenase processing or activity. In general, the rate of hydrogenase activity was dependent on the level of hydrogenase structural genes estimated by the analysis of either *hup-lacZ* expression or HupL protein content.

This work was supported by CICYT (BIO93-0046) and EC CT930053 (IMPACT) Projects.

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TWO DIFFERENT SIGNAL PEPTIDASES OF *BRADYRHIZOBIUM JAPONICUM*, SPECIFICALLY REQUIRED IN SYMBIOSIS WITH SOYBEAN.

MÜLLER P., AHRENS K., JACOBI A., KELLER T., KLAUCKE A., WERNER D.
Philipps Universität, FB Biologie, Karl-von-Frisch-Str., 35032 Marburg, Germany.

Using Tn*phoA* for a general mutagenesis of *Bradyrhizobium japonicum* 110*spc*4 several mutant strains were obtained which were disturbed specifically in their symbiotic interactions with soybean (*Glycine max*). Two of them, strains 132 and 184, were analyzed in detail because they performed novel symbiotic phenotypes whereas their *ex planta* nitrogenase activity was still intact (Müller et al., 1995). A single Tn*phoA* insertion in mutant 132 resulted in enhanced alkaline phosphatase activity, indicating the formation of a functional fusion peptide which was secreted out of the cytoplasm. Using a PhoA antiserum in a Western analysis revealed that in mutant 132 a faint protein band of 70 kD and a prominent band of 52 kD were detected which both were missing in the wild type strain. The mutated DNA region was cloned and the corresponding 4.4 kb *Bam*HI wild-type fragment was sequenced. These data confirmed that '*phoA* was fused in frame to ORF2 (269 aa) homologous to the *E. coli lep* gene, encoding a type I signal peptidase. It was found that the deduced amino acid sequence of the fusion protein fits well will the 70 kD band, whereas the 52 kD protein is slightly bigger than the PhoA moiety. The temperature-sensitive *E.coli lep* mutant IT41 (Inada et al., 1989) is genetically complemented by a *B. japonicum* wild-type DNA fragment carrying the homologous gene. Therefore the *B. japonicum* gene product is functional also in *E. coli*. The inactivation of ORF3 (334 aa) further downstream by an *aphII* resistance cassette showed that this gene product was not required for complementation of the *E. coli* lep^{ts} mutant and its inactivation did not cause a symbiotic phenotype in *B. japonicum*. Therefore a polar effect of the Tn*phoA* insertion in strain 132 can be excluded. Further mutational and complementation analyses indicate that the operon ends with ORF3 and that the promoter of this operon is located upstream of ORF1. The functions of ORF1 and ORF3 remain to be determined. Soybean nodule cells infected with mutant 132 are highly vacuolized and the bacteroids are rapidly lysed soon after release from infection threads. Using specific antibodies directed against symbiosome membrane proteins revealed a lower concentration of these nodulins in mutant induced nodules.

The symbiotic phenotype of mutant 184 is characterized by an abnormal pattern of colonization. Sequence analysis of the DNA region that has been tagged by Tn*phoA* indicates the presence of a second type I signal peptidase in *B. japonicum* 110*spc*4.

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Müller et al. (1995) Planta, in press

A NOVEL SENSOR PROTEIN OF *BRADYRHIZOBIUM JAPONICUM* REQUIRED FOR THE SYMBIOTIC INTERACTION WITH SOYBEAN.

MÜLLER P., KLAUCKE A., WERNER D. (1),
THÖNY-MEYER L., HENNECKE H. (2)
(1) Philipps Universität, FB Biologie, Karl-von-Frisch-Str., 35032 Marburg, Germany.
(2) ETH Zürich, Mikrobiologisches Institut, Schmelzbergstraße 7, 8092 Zürich, Switzerland

A Tn*5*-induced mutant of *Bradyrhizobium japonicum* 110*spc*4 (strain 3652) is disturbed specifically in its symbiotic interactions with soybean (*Glycine max*), whereas under *ex planta* conditions nitrogenase activity is not affected (Regensburger et al., 1986).

Nodules induced by mutant 3652 are characterized by an instability of the symbiosome membrane, bacteroid deformation and degradation. DNA sequencing revealed that Tn*5* is inserted within a large ORF, designated *bdfA* (*b*acteroid *dif*ferentiation). *bdfA* apparently is cotranscribed with two other ORFs further upstream, which are homologous to *dfp* (*D*NA-synthesis *f*lavo*p*rotein) and *dut* (*d*esoxy*u*ridine-*t*riphosphatase). In *E. coli* it has been shown that both gene products are involved in pyrimidine biosynthesis (Spitzer, Weiss, 1985).

Using a 1.5 kb *Eco*RI fragment, directly upstream of *bdfA* and coding for the C-terminal part of Dfp and for Dut, for vector integration mutagenesis, resulted in a disrupted operon. As a consequence, *bdfA* was expressed not at all, since no indigenous promoter was present on the introduced DNA-fragment and the plasmid-borne p*lac* promoter was oriented in the opposite direction. The symbiotic phenotype of the resulting mutant VIM-105 is characterized by an impaired release of the mutants from the infection thread, enlarged peribacteroid spaces filled with electron dense material, and a poor colonization of the central nodule tissue.

The deduced amino acid sequence of *bdfA* shows a high degree of sequence homology to other procaryotic sensor proteins. The highest scores were obtained with DivJ and PleC, two histidin protein kinases of *Caulobacter crescentus* which are involved in cell differentiation and cell division (Ohta et al., 1992). The organisation of *dfp*, *dut* and *bdfA* in one operon suggests, that bacteroid differentiation is tightly coupled with the cell cycle of *B. japonicum*.

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RHIZOBIUM MUTANTS WITH CHANGED NITROGEN AND COLD RESISTANCE AND LEUCINE AUXOTROPHY

NICHIK M.M., YERKO V.N., KOTS S.YA.,
MANDROVSKAYA N.M., STARCHENKOV E.P.
Institute of Plant Physiology and Genetics, National Academy of Sciences of Ukraine, Kiev, 252022, Ukraine

The development of symbiosis between *Rhizobium* and legumes depends on many factors and is regulated both by the genes which participate in the formation only of symbiotic apparatus and by the genes of general metabolism. Therefore it is very important to study the genes which influence the formation of symbiosis in indirect way. *R.meliloti, R.leguminosarum* and *Bradyrhizobium sp.* (*Lupinus*) mutants with changed symbiotic properties have been obtained. *R.meliloti* strain M4 revealed the heightened efficiency in the presence of mineral nitrogen. The application of 3mM of nitorgen per kg of soil resulted in the increase of nitrogen fixation, photosynthetical and respiratory intensity of the plants, inoculated by this strain in comparison with the strain-standart. A higher nitrate reductase activity was found in lucerne stems when inoculating by M4 strain against control. In this case the surplus of green mass made up 46%. Cold resistant mutants (*B.sp.(Lupinus)* and *R.leguminosarum*) formed pink nodules at 8-12°C. *B. sp.* (*Lupinus*) formed nitrogen fixing nodules by 7-10 days and *R.leguminosarum* - by 3-5 days earlier than wild type of strains. Symbiotic nitrogen fixation when inoculating by mutants increased by 16-60% and surplus of plant mass was 13-15% against control. Among Tn5 auxotrophic mutants of *Rhizobium meliloti* SAM1 we identified the mutant with a block in leucine biosynthesis pathway. Addition of leucine to lucerne plants inoculated by *R.meliloti* leucine auxotroph did not cause the restoration of symbiosis. The investigation of nodules formed by the plants inoculated by this leucine auxotroph has shown that mutant nodule bacteria penetrating in root hair formed infection threads. However, rhizobia did not release infection threads and perished within them. Thus, *R.meliloti, R.leguminosarum* and *B.sp.(Lupinus)* mutants with changed and in some cases improved symbiotic properties have been obtained. The investigation of these mutants is still in progress.

REGULATION AND FUNCTION OF GENES IN *RHIZOBIUM MELILOTI* INDUCED IN RESPONSE TO TEMPERATURE DOWNSHIFT

O'CONNELL K[1]. GUSTAFSON A.[1,2], LEHMANN D.[1,3],and
THOMASHOW, M.[1,2,3]
[1]Center for Microbial Ecology and Departments of [2]Microbiology and [3]Crop and Soil Science, Michigan State University, East Lansing, MI 48824 USA

Soil microorganisms commonly experience a variety of environmental stresses such as extremes in temperature, drought, and nutrient deprivation. Little is known in any organism about either the roles that cold-regulated genes have in low temperature tolerance or the mechanisms of cold-regulated gene expression. We identified two mutants that form colonies on solid media more slowly than wild-type strain RM1021 at 15°C but not at 30°C. Sequences flanking the insertion in mutant RM28 showed no significant identity with sequences in the GenEMBL database. The insertion in mutant RM43, however, resides in a gene with significant similarity to *tktA*, which encodes transketolase. RM43 grows poorly at 30°C on minimal medium. In addition, RM43 remains culturable after prolonged incubation at 2°C, whereas RM1021 does not. We identified genes induced by temperature downshifts by mutagenizing the RM1021 genome with a *luxAB* reporter transposon. We isolated nine mutants whose light emission increased 15- to 150-fold. Sequencing the DNA flanking the transposons revealed that four mutants have insertions in putative open reading frames with no significant identity to sequences in the GenEMBL database, suggesting that we may have identified novel genes induced in response to low temperature. The four other mutants have insertions in rRNA genes (two each in 23S and 16S). Surprisingly, none of the mutations are in genes known to be induced by cold-shock in *E. coli* (Jones, Inouye 1994). Preliminary experiments suggest that sequences required for temperature downshift regulation of a 16S rRNA gene are within 600 bp of the putative rRNA transcriptional start site. Increases in the rate of rRNA synthesis in *E. coli* after cold shock have not been reported; therefore, the observation that an rRNA operon(s) is induced after temperature downshift may represent the discovery of a novel temperature adaptation mechanism in bacteria. We are currently investigating the contributions of these genes to growth and competitiveness at low temperatures, and identifying regulatory elements involved in sensing decreases in temperature.

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ROLE OF NITROGEN AND A *glnD* HOMOLOG IN INDUCTION OF CHLOROSIS IN BEANS BY *RHIZOBIUM TROPICI*

O'CONNELL, K.[1], STEWART, S.[2], SAVILLE, B. J.[3], and HANDELSMAN, J.[2]
[1]Center for Microbial Ecology, Michigan State University, East Lansing, MI 48824 USA, [2]Department of Plant Pathology, University of Wisconsin-Madison, Madison, WI 53706 USA, [3]Biology Group-Erindale College, University of Toronto, Mississauga, Ontario, L5L 1C6 Canada.

Type B strains of *Rhizobium tropici* induce severe chlorosis in the leaves of symbiotic host and non-host dicot plants (O'Connell et al. 1990, O'Connell and Handelsman 1993). The addition of carbon substrate to the rhizosphere is required for this activity. Chlorosis induction by strain CIAT899 can be prevented by a high N:C ratio in the rhizosphere. We have isolated a Tn5 insertion mutant of CIAT899, designated CT4812, that no longer induces chlorosis in bean plants. We have also identified a cosmid, pJM528, that contains CIAT899 DNA and restores chlorosis-inducing ability to CT4812. Subcloning and sequencing a 3.0 kb fragment of pJM528 that also restores chlorosis induction reveals that the Tn5 insertion in CT4812 is located in an open reading frame (ORF) with 31%/56% amino acid sequence identity/similarity to *glnD,* which encodes uridylyltransferase/uridylyl-removing enzyme in *E. coli,* and 34%/54% amino acid sequence identity/similarity to *nfrX,* a similar gene in *Azotobacter vinlandii* (Contreras et al. 1991) Hybridization analysis indicates that this gene resides on the chromosome of CIAT899. When the genomic DNA of several *R. tropici* strains was probed with the *glnD*-like sequence of CIAT899, all type B strains except one shared an identical hybridization pattern that differed from the pattern shared by type A strains. Immediately downstream of the *glnD*-like ORF in CIAT899, we have sequenced part of an ORF with significant amino acid sequence similarity to *mviN,* a virulence factor in *Salmonella typhimurium.* Cosmid pJM528 did not confer chlorosis-inducing ability on other species of bacteria. The transposon insertion and flanking DNA in CT4812 were cloned into the plasmid pSUP202, and subsequently introduced into CIAT899. The resulting marker exchange mutant, CT667, failed to induce chlorosis in plants and grows very poorly on nitrate as the sole source of nitrogen. We are currently working to determine whether the *glnD*-like gene in CIAT899 is functionally homologous to *glnD.*

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GENOMIC REARRANGEMENTS THAT ENHANCE THE SYMBIOTIC EFFECTIVENESS OF *Rhizobium etli*.

ROMERO, D.[1], VALDERRAMA, B.[2], PALACIOS, R.[1], EPARVIER, A.[1]
AND RODRIQUEZ, C.[1]
Depts. of Molecular Genetics1 and Molecular Ecology2, CIFN-UNAM.
Ap. Postal 565-A, Cuernavaca, Mexico

The *Rhizobium* genome is prone to the frequent formation of rearrangements. To identify possible beneficial effects of these rearrangements,we have characterized a collection of strains carrying different alterations in the p*Sym* . In this work, the starting strain was DEM151, a derivative of *R. etli* CFN42 carrying an interposon in *nifHDK* region a. DEM151 is 50% less effective than strain CFN42, evaluated by specific nitrogenase activity (SNA) and plant dry weight (DW) measurements in greenhouse experiments. From DEM151, we have selected a spontaneous rearrangement that translocates a 170 Kb *nod-nif* sector from the p*Sym* into an endogenous plasmid (plasmid pa, see Corvera et al., this congress),thus giving rise to plasmid pGM1. A strain harboring pGM1 (CFNX250) has an improved symbiotic effectiveness, with values for SNA and DW similar to CFN42, despite the presence of the original mutation in *nifHDK* region a. Further improvement in the effectiveness of CFNX250 was achieved through the tandem duplication of a 120 Kb *nod-nif* region in pGM1. This strain (CFNX253) gave values for SNA and DW 30 to 40% higher than those with CFN42.

This work suggests a new strategy for the improvement of symbiotic effectiveness, taking advantage of the cell's ability to manipulate large DNA regions *via* genomic rearrangements.

MELANIN PRODUCTION IS CONTROLLED BY THE MEGAPLASMID 2 IN *RHIZOBIUM MELILOTI* STRAIN CA15-1.

SHARYPOVA L.A., ONISHCHUK O.P., SAGULENKO E.A., EVDONIN A.L., CHIZHEVSKAYA E.P.
Research Institute for Agricultural Microbiology, Podbelsky Shosse, 3
St.Petersburg-Pushkin 8, 189620, Russia

Melanin production was reported for many strains of root-nodule bacteria. This pigment is synthesized by the enzyme tyrozinase (EC 1.14.18.1). However its biological significance in the *Rhizobium*-legume symbiosis is not well understood. Up to date, genes controlling *mel*-production were identified in a few strains of *R.leguminosarum bv.phaseoli* (on *Sym*-plasmids) and *R.l.bv.viceae* (on *Sym*- and cryptic plasmids); and in *R.meliloti* strain GR4 (on cryptic plasmid) (Beynon et al., 1980, Hynes et al., 1984, Mercado-Blanco et al., 1993).

Some of *R.meliloti* strains isolated from Central Asia (CA-strains) were shown to have an ability to produce melanin on TY medium in the presence of tyrosine and $CuSO_4$. The aim of the work was to study genetic determination of this property in *R.meliloti* CA-strains.

Strains CA15-1 and CA15-2 were initially isolated from the culture of nodule isolate 15-1 due to their difference in polysaccaride production. Strain CA15-2 (Mel^- phenotype) in contrast to CA15-1 (Mel^+) was found to contain a prophage, which was isolated by the irradiation of bacterial culture with UV-light. When CA-strains were lysogenized with the phage ϕCA15-2, 3 out of 9 non-producing strains gained Mel^+ phenotype. Evidently phage ϕCA15-2 is able to cause either phage conversion of *R.meliloti* strains or specific transduction of a gene, involved in the melanin production.

Strain 15-1 contains 2 megaplasmids (M1 and M2) and one cryptic plasmid of 260 kb. When M2 of 15-1 was replaced by the M2 of laboratory strain CXM1-188, obtained transconjugants lost ability to produce melanin. This evidenced that Mel^+ phenotype was encoded by the M2 of strain CA15-1.

To construct strain, carrying genetic marker linked to *mel*-gene, 5 different *Tn*5-mob insertions from M2 of CXM1-188 we transferred by the use of phage φM12 into strain CA15-1. This allowed to isolate a recombinant in which distance between *mel*-gene and *Tn5-mob* was estimated as 0,6 kb. To study expression of *mel*-15 in CXM1-188 background, we cotransduced it with *Tn*5-mob. None of 150 transductants showed Mel^+ phenotype. Consequently, *mel*-15 is not the only gene involved in pigment production in strain CA15-1.

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GENETIC STUDIES ON UNICELLULAR N_2-FIXING CYANOBACTERIA

SHESTAKOV, S. (1), KAMILOVA, F. (2), MIKHEEVA, L. (1), GRIGORIEVA, G. (1), BELAVINA, N. (1), SHAKHNABATIAN, L. (1), ERNST, A. (3), BÖGER, P. (3), STAL, L.J. (4) AND GALLON, J.R. (5).
(1) Department of Genetics, Moscow State University, Russia.(2) Institute of Genetics, Tashkent, Uzbekistan.(3) Department of Plant Physiology and Biochemistry, University of Konstanz, Germany.(4) Laboratory for Microbiology, University of Amsterdam, The Netherlands.(5) Biochemistry Research Group, University of Wales, Swansea, U.K.

Certain unicellular cyanobacteria are able to perform both N_2 fixation and oxygenic photosynthesis within a single cell. In order to study, at the molecular level, the regulation of N_2 fixation and the mechanisms of protection against oxygen, we have isolated mutants of *Gloeothece* spp PCC 6909 and CCAP 1430/3 (strains capable of aerobic N_2 fixation) and *Synechocystis* sp BO 9201 (a strain that fixes N_2 only microaerobically) and have started to develop reliable gene transfer systems in these cyanobacteria.

A collection of mutants resistant to agents inducing oxidative stress (paraquat, plumbagin, menadion, amitrol) has been obtained. Some of such *Synechocystis* BO 9201 mutants are characterised by alterations in Photosystem II/ Photosystem I and in phycoerythrin/phycocyanin ratios. Among *Gloeothece* mutants altered in nitrogen metabolism or resistant to oxidative stress we have found a number of strains which are incapable of aerobic nitrogen fixation (acetylene reduction). These mutants form yellow colonies on N-free agar medium but normal dark-green colonies on media supplemented by ammonium or nitrate.

A cosmid genomic library of *Synechocystis* BO 9201 has been constructed on the basis of plasmid Lorist 6. The average size of cyanobacterial DNA inserts is about 40 kb. A systems for gene transfer by conjugation and electrotransformation have been developed for this cyanobacterium. Plasmid vectors, based on the pDU1 and RSF1010 replicons modified by copropogation with helper plasmid carrying the methylase gene are now available for genetic analysis and gene cloning in *Synechocystis* BO 9201. Unfortunately these plasmids do not replicate in *Gloeothece* cells which seem to possess some unidentified restriction endonucleases that prevent the maintenance of these vector plasmids.

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STUDY OF THE STRUCTURE AND STABILITY OF THE RECOMBINANT PLASMID PRM46 WHICH HARBOURS *NOD-HSF* GENES OF *RHIZOBIUM MELILOTI* 1774, ISOLATED FROM *MEDICAGO ARABICA* NODULES.

SIMAROV, B.V., RUMYANTSEVA, M.L., KROL, E.A., YAKUTKINA, V.V., ANDRONOV, E.E. Research Institute of Agricultural Microbiology, St.-Petersburg-Pushkin, Russia

R.meliloti 1774 is a Rf^r derivative of the wild-type strain 135a which was isolated from the nodules of the annual plant *Medicago arabica* in St.-Petersburg region (Vasjuk, 1979). This strain formed an effective symbioses not only with *M.arabica* but also with *M.varia*. In contrast, the majority of *R.meliloti* wild-type strains are symbiotically effective with *M.varia*, but form only non N_2-fixing root nodules on *M.arabica*. We assumed that *R.meliloti* 1774 contained new symbiotic genes - *hsf* - controlling host-specificity of nitrogen fixation on *M.arabica*. Since many sym genes (*nod, nif*) are located on the pSym of *R.meliloti* strains investigated to date (Denarie, 1992), we proposed that the *hsf* genes of strain 1774 are also located on the pSym1774b.

Plasmid pSUP5011 (Tn5-mob; Simon, 1984), was chosen for the general Tn5 mutagenesis of *R.meliloti* 1774 with the intention of marking the pSym1774b by Tn5-mob and then transferring it to non-nodulating *R.meliloti* CXM1-46 recipient (Sm^r UV-mutant of wild-type CXM1; Feodorov, Simarov, 1987). *R. meliloti* CXM1 formed effective symbiosis with *M.varia* and non-fixing white nodules with *M.arabica*. The resulting 114 transconjugants were analysed in a plant test. The restoration of the nitrogen fixing phenotype in symbiosis with *M.varia* was determined in 18 transconjugants and two of them - TR46-53 and TR46-58 - formed effective symbioses with *M.arabica* and *M.varia*. It was found that all 18 transconjugants contained a new recombinant plasmid - pRM46 (Tra+) of 92,6 kb. The physical map of pRM46 was determined on the basis of *EcoR*I and *Pst*I restriction and blot hybridization analyses with several sym genes probes. It was conclude that the use of RP4/pSUP5011 cointegrate formation (Simon et al., 1986) enabled us to clone *in vivo* 18 kb region of pSym1774b. The presence of *nod*ABC-*nod*GEFH region on the pRM46 was shown. The fact that the transconjugants TR46-53 and TR46-58 were able to form effective symbioses with *M.varia* and *M.arabica* proved that the cloned 18 kb region contained the *hsf* genes. The analysis of this region and the cloning of the *hsf* genes of *M.arabica* are currently under investigation.

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RHIZOBIUM ETLI MUTANTS ARE CAPABLE OF EXPRESSING C-TYPE AND AA3 CYTOCHROMES UNDER CERTAIN CULTURE CONDITIONS

TABCHE ML[1],GARCIA E[2], MIRANDA J[1],ESCAMILLA E,[2]
SOBERON M.[1]
[1] Instituto de Biotecnologia UNAM Apdo Postal 510-3, Cuernavaca,
Morelos 62271
[2] Instituto de Fisiologia Celular, Departamento de Microbiologia
UNAM Mexico D.F.

A *Rhizobium etli*, IFC01 unable to oxidase N,N,N',N'-tetrametil-*p*-phenilenediamide (TMPD) was isolated and charaterized. The cloning and sequence of the mutated gene reveled that the Tn*5mob* insertion was within a gene highly homologous to the *Bradyrhizobium japonicum* (Ritz et al., 1993) and *Rhizobium meliloti* (Kerest et al ., 1995) *cycH* genes involved in c-type cytochrome biogenesis. By sequence analysis,it was also identified an homologous *cycJ* gene localated to the 3' end of *cycH* gene. We found that a previosly described *R.etli*, cytochrome c-deficient mutant (CFN4202) (Soberon et al., 1993) that induced empty nodules on *Phaseolus vulgaris*, was also affected in the *cycH* gene. Nevertheless the *cycH* mutant described in this paper (IFC01) was able to infect bean plants, but unable to fix nitrogen during symbiosis. Stationary cultures of IFC01 strain showed no c-type and aa_3 cytocrhomes, nevertheless, both types of cytochromes were expressed in exponential cultures of this strain. Finally evidence is presented suggesting that the *cycJ* gene, but not *cycH*, is reitered in *R. etli.*.

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TRANSCRIPTIONAL REGULATION OF REITERATED NITROGENASE GENES IN Rhizobium etli.

VALDERRAMA, B., DAVALOS, A., MORETT, E.*, AND MORA, J.,
Departamento de Ecología Molecular, Centro de Investigación sobre Fijación
de Nitrógeno and * Departamento de Reconocimiento Molecular y Bioestructura,
Instituto de Biotecnología, Universidad Nacional Autónoma de México,
AP 565-A, Cuernavaca, Morelos, México.

Reiterated nitrogenase reductase (nifH) genes are common among Rhizobium species. R. etli strain CFN42 has three identical copies denominated a, b and c (Quinto et al, PNAS 82:1170, 1985). Copies a and b are transcriptionally linked to the nitrogenase structural genes (nifDK), while no nifDK genes have been identified downstream of copy c. To achieve full nitrogenase performance during symbiotic nitrogen fixation with bean plants, both nifHDK operons are required, while insertions in the third copy do not present any symbiotic phenotype (Romero et al, Appl Env Microb 54:848, 1988). Eventhough, the three copies are actively expressed in nodules (Morett et al, MGG 213:499, 1988). In this work we analyse the free living transcriptional regulation of these reiterated nifH genes. Sequencing the downstream region from copy c we found three open reading frames. ORF70 codes for the amino terminal portion of a NifD protein (100% identical to the nifD gene from copy b until residue 196, where identity ends). We have no evidences of its functionality.

We constructed nifH::lacZ fusions in regions a and c and measured their relative free living expression levels at different oxygen concentrations. Our results indicate that in their natural background, the nifHc gene is expressed three times as much as the nifHa gene. Both genes are induced specifically at 1% oxygen concentration. Symbiotic relative activities are consistent with these data, since nifHc is expressed at a higher level than nifHa, despite it is not functional.

Upstream from the three nifH genes are located sequence resembling highly consensus RpoN dependent promoters (GGCACGGGTTTTGA) activated by the ReNifA protein. In regions a and b, there are canonical putative NifA binding sites 88bp (TGT-n10-ACA) upstream of the promoters, while nifHc has two imperfect putative NifA binding sites located 86bp (TGG-n10-ACA) and 168bp (TGT-n10-ACG) upstream of the promoter.

Heterologous expression experiments demonstrate the requirement of EcoRpoN and KpNifA for the transcription of both genes. A mutant form of KpnNifA unable to bind DNA did not activate the transcription of copies a and c.

In vivo DMS footprinting analyses showed that KpnNifA binds to the cognate UAS and to a TGT UAS half site at position -112 in copy a. In region c, KpnNifA protected nucleotides -95, -96, -98 and -99, belonging to the promoter proximal imperfect UAS. No protection was detected at the distal imperfect UAS. We therefore conclude that these elements are responsible for the differential expression of the copies a and c.

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HOXA IS A TRANSCRIPTIONAL REGULATOR OF HYDROGENASE ACTIVITY IN *BRADYRHIZOBIUM JAPONICUM*

C. VAN SOOM AND J. VANDERLEYDEN
F. A. Janssens Laboratory of Genetics, Willem de Croylaan 42, B-3001
Heverlee, Belgium.

Bradyrhizobium japonicum forms nitrogen-fixing nodules on the roots of its host plant, soybean. During symbiotic nitrogen fixation, hydrogen is produced as an obligate by-product of the nitrogenase reaction. Some rhizobia possess an hydrogen-uptake (Hup) system that re-oxidizes the hydrogen in nodules. In Hup^+ *Bradyrhizobium japonicum* strains, hydrogenase activity can also be induced in free-living conditions.

The *B. japonicum hoxA* gene is homologous to transcriptional regulators of the NtrC family (Van Soom et al. 1993). To investigate a possible regulatory function of the *hoxA* gene product, we constructed a *hoxA* mutant and evaluated its Hup phenotype in free-living and symbiotic cells. In free-living conditions, the *hoxA* mutant had no active hydrogenase. In symbiotic conditions, HoxA mutants only had 50% of the wild-type Hup activity. In accordance with these results, the expression of the hydrogenase structural genes *hupSL* was severly impaired in a *hoxA* mutant under hydrogenase-inducing conditions.

The structural characteristics of HoxA suggest that it is a transcriptional activator which acts through DNA binding. Therefore, we examined whether HoxA exerts its regulatory function by binding at the promoter region of the *hup* structural genes, by means of gel shift experiments. In crude protein extracts isolated from *B. japonicum* cells grown heterotrophically or induced to express hydrogenase activity, a protein was detected that specifically binds at the *hup* promoter region. No binding is observed when protein extracts from the *hoxA* mutant are used. When we overexpressed and purified HoxA in the form of a Maltose-Binding-Protein/HoxA fusion protein (MBP-HoxA), this fusion protein also specifically binds at the *hup* promoter region.

We conclude that the HoxA protein binds at the *hup* promoter region, in accordance with its structural characteristics. Combined with the *hoxA* mutant phenotype and expression experiments, these data provide evidence for the regulatory role of HoxA in modulating the expression of the *hup* structural genes.

Van Soom et al. (1993) Mol. Gen. Genet. 239, 235

A NOVEL SYMBIOTIC REGION IN THE GENOME OF *RHIZOBIUM LEGUMINOSARUM*

YEOMAN, K.H. [1], DELGADO, M-J [2], DOWNIE, J.A. [2] AND JOHNSTON, A.W.B.[1]
[1] University of East Anglia, Norwich NR4 7TJ, UK
[2] John Innes Centre, Norwich NR4 7UH, UK

During nodule development, rhizobia differentiate into N_2-fixing bacteroids. Low oxygen concentration (3-30 nM) is needed for the optimal functioning of the O_2-sensitive nitrogenase enzyme. One aspect of bacteroid differentiation is the development of a specialized branch of the respiratory pathway, terminating with an oxidase with a high affinity for O_2. In *Bradyrhizobium japonicum* , a gene cluster, *fixNOPQ*, encodes *c*-type cytochromes and a membrane-bound cytochrome *c* oxidase in for this pathway.

Mutants of *Rhizobium leguminosarum* biovar *viciae* which were unable to respire *via* the cytochrome aa_3 pathway were identified by their inability to oxidise NN'dimethyl *p* phenylene diamine. Two mutants were complemented by a cosmid pIJ1942, isolated from a *R.leguminosarum* clone bank. Pea nodules induced by these mutants contained many bacteroids which were ineffective for nitrogen fixation. The mutants lacked spectroscopically detectable cytochrome *c* . Heme staining of the cellular proteins showed the absence of all cytochrome *c*-type heme proteins. A component attributed to cytochrome c_1 was still present in an inner membrane fraction. Cytochromes aa_3 and *d* were present, the latter at higher than normal levels. DNA sequence analysis of the complementing plasmid showed four apparently co-transcribed open reading frames (*cycH, cycJ, cycK* and *cycL*) which are homologous to the corresponding genes in *B. japonicum* and *R.meliloti*. These gene products are thought to be involved in the attachment of heme to the cytochrome *c* apoprotein. CycK and CycL are homologous to the *Rhodobacter capsulatus ccl*1 and *ccl*2 gene products, and also to the *Escherichia coli nrf*E and *nrf*F gene products involved in the assembly of *c*-type cytochromes.

It was noted that *cycH* is preceeded by another gene in the same orientation and with a product whose sequence is typical of membrane-bound lipoproteins. Further, immediatley upstream of this "*lip*" gene are two other genes termed *popP* and *popQ* which appear to encode proteins homologous to bacterial two-component transcriptional sensor regulator systems.

Coincidently an overlapping cosmid of pIJ1942 was also isolated, termed pKN16. This cosmid was able to correct a mutant of *Rhizobium leguminosarum* which was defective in high affinity iron acquisition. The mutant induced nodules on peas but the bacteroids were ineffective for nitrogen fixation. The cytochrome *c* and *b* content, determined spectroscopically was reduced, and the mutant also accumulated protoporphyrin IX a precurser of heme.

Another striking feature of the DNA in pKN16 was that it contained a cloned gene whose product had homology to eukaryotic [particularly plants] glutathione-S-transferases. This enzyme has a wide variety of activities; most notably, it has a key role in detoxifying a range of xenobiotics such as herbicides in plants and certain carcinogens in mammalian liver. Strikingly, the *gst* gene of *Rhizobium* was more similar to that in plants than to other *gst*-like genes found in other bacteria.

THE STUDY OF LEUCINE AUXOTROPH OF *RHIZOBIUM MELILOTI*

YERKO V.N.
Laboratory of Molecular Genetics, Institute of Plant
Physiology and Genetics, Kiev, 252022, Ukraine

At present the new information appeared on participation of genes of amino acid biosynthesis in nitrogen fixation (Aguilar et al., 1991). It is known that leucine auxotrophs of *Rhizobium meliloti* do not form symbiosis with alfalfa (Denarie et al., 1975). However, the symbiosis is restored when alfalfa plants are supplied with exogenous leucine (Truchet et al., 1980). With the use of *Tn5* mutagenesis the leucine auxotroph of strain *R.meliloti* SHM1 was obtained, which did not restore the symbiosis if leucine was added to plants inoculated with this auxotroph (Aronshtam et al., 1993). So the investigation of this leucine auxotroph might be interesting for the elucidation of participation of leucine biosynthesis enzymes in the formation of symbiosis. Therefore, from parent strain *R.meliloti* SHM1 the EcoRI fragment of 4,5kb has been cloned, and this fragment entirely complemented both as auxotrophy and symbiotic properties of the mutant. A successful complementation of leucine auxotrophy of this mutant by another gene bank, ***R.leguminosarum*** bv.*viceae* VF39, did not however result, in the restoration of symbiotic properties within prototrophyc transconjugants. The explanation of the results obtained can't be considered definitively. The investigation of this leucine locus is now in progress.

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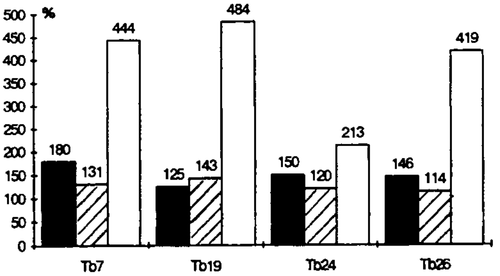
STUDY OF *R.MELILOTI* MUTANTS WITH INCREASED RESPIRATORY ACTIVITY

YURGEL, S.N(1), SHARYPOVA, L.A. (1), SYRTSTOVA, L.A. (2), SIMAROV, B.V. (1).
(1) Research Institute for Agricultural Microbiology, Podbelsky Sh.3, Pushkin-8, St.Petersburg, 189620, Russia.
(2) Institute of Chemical Physics of Russian Academy of Sciences, Moscow Region, Chernogolovka, 142432, Russia

We obtained a collection of 28 *Tn*5-mob induced mutants in *Rhizobium meliloti* CXM1-188 with increased 2,3,5-triphenyltetrazolium (TTB) bromide reducing activity. They were named Red^{++}. Symbiotic properties of Red^{++} mutants were tested under gnotobiotic conditions on *Medicago sativa cv.Wega*. Nodules, formed by 24 Red^{++} strains, fixed 23-80% more N_2 than did nodules formed by the strain CXM1-188, as judged by the total nitrogen content found in the inoculated plants. By the use of conjugation we mapped 23 mutations to the megaplasmid 2 and 5 - to the chromosome. To estimate how many loci were affected by the *Tn*5 in Red^{++} mutants, we hybridized their total DNA with DIG labeled plasmid pLS7, carrying *Tn*5. This allowed to distinguish 9 different loci on megaplasmid 2 and 5 - on the chromosome. We estimated respiratory activity of the mutants by the rate of CO_2 production in static cultures. Twenty out of 23 mutants were found to produce significantly more CO_2 than the parental strain. In addition, we isolated bacteroids from nodules of alfalfa plants infected with 4 different mutants, and tested their respiratory activity. It was found that all Red^{++} mutants had significantly higher succinate and HADH oxidase activity than strain CXM1-188 (Fig.1). By this means *Tn*5 mutants with Red^{++} phenotype showed enhanced symbiotic effectiveness and higher respiratory activity in bacteroids and in free-living cultures.

Fig.1 Symbiotic and respiratory activity of Red^{++} mutants



■ total nitrogen in plant shoots (% to CXM1-188); ▨ respiration rate in free-living cells (% to CXM1-188); □ respiration rate in homogenates of bacteroids (% to CXM1-188)

Acknowledgments. This work was supported the Russian State Program "Frontiers is Genetics", and by the International Project "Interbioazot-2000"

RHIZOBIUM MELILOTI MUTANTS WITH ALTERED SURFACE AND THEIR SYMBIOTIC PROPERTIES

ZATOVSKA T.V.(1), KOSENKO L.V.(1), USHAKOV K.V.(2), YURGEL S.N. (2), SIMAROV B.V.(2)
(1) - Institute of Microbiology and Virology, National Academy of Sciences of Ukraine, Zabolotny St., 154, Kiev-143, Ukraine.
(2) - Recearch Institute for Agricultural Microbiology, Sh.Podbel sky 3, St.Petersburg - Pushkin 8, 189620, Russia.

During establishment of symbiosis between *Rhizobium* and legumes surface components of bacterial cell play important role. Lipopolysaccharides (LPS) were thought not essential for symbiosis because there are number of examples that mutants with altered LPS were not defective in symbiosis (Clover et al, 1989). But report that mutant of *R.meliloti* with changed LPS had a significantly decreased competitiveness for nodulation in alfalfa (Lagares et al, 1992) discovered a new view on importance of LPS for succesful symbiosis.

To understand the role of LPS in symbiosis between *R.meliloti* and alfalfa we studied *Tn5*-mutants with altered LPS. LPS-mutants of *R.meliloti* str. CXM1-188 were screened on the medium LB containing sodium deoxycholate (DOC) and media with Congo Red. We isolated 5 mutants with different changes in cell surface. Two mu tants- *Rm19* and *Rm22* - did not grow in presence DOC and had altered colour on medium with Congo Red. Three other mutants - *Rm10*, *Rm12* and *Rm152* - differed from parental strain on medium with Congo Red. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of LPS demonstrated the absence of LPS1 for *Rm19* and *Rm22* and modificated LPS1 for *Rm10*. Analysis of transductants, obtained with phage ϕ M12 for each mutant, demonstrated identity of LPS-mutants and their transductants in their cultural properties and LPS profiles.

The symbiotic properties of mutants and transductants were studied on alfalfa (*Medocago sativa cv. Vega*) under sterile conditions. We estimated competitiveness by indirect method using a Fix$^-$ mutant as a reference strain. All mutants had Fix$^+$ phenotype. *Rm152* and its transductants resulted in increase of plant mass. Mutants *Rm19* and *Rm22* and its transductants which had no LPS1 were defective in competitiveness. The *Rm12* and *Rm152* and their transductants had delayed competitiveness. Competitiveness of *Rm10* had no difference from parental strain. Our results evidence that cell surface alterations may influence on symbiotic phenotype of *R.meliloti*.

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CLONING AND SEQUENCING OF A 3. 7 KB ENHANCING FACTOR FROM *RHIZOBIUM FREDII* B52

ZHANG, X.,ZHOU, J., ZHANG, Z., MA, H.S.L. AND CHEN, H.
Department of Mcirobiology, Huazhong Agriculutural University,
Wuhan, 43007, P.R. China.

A new method has been designed to construct high effective strains: firstly, we constructed the gene library of *Rhizobium fredii* strain B52 genome using pLAFR1 as the vector; then transferred this library into *Bradyrhizobium japonicum* strain 22-10 by triparvent mating; subsequently, mixed all transconjugants to inoculate soybean, using the plant as the selective agent for screening transconjugants with enhanced symbiotic effectiveness; finally, a high effective strain, HN32, was obtained and has been widely used as soybean inoculant in Hei-LongJiang、Sichuan and Guaxi Provinces with the average of 11. 4% yield increase.

The recombinant plasmid pHN32 in strain HN32 contains a 3. 7kb insert called enhancing factor. In order to analyse its nucleotide sequence, a 0. 5kb EcoRI fragment from pHN32 was cloned into M13mp18 in both orientations. A 3. 2kb EcoRI fragment of pHN32 was cloned into pUC19 in both orientations. Then a nest of deletions were generated from both orientations using exonuclease Ⅲ. All subclones were sequenced by Sanger method using Taq DNA polymerase. Finally, sequences of individual subclones were merged by means of the Microgenic software (Beckman). Two open reading frames(ORF$_1$ and ORF$_2$) was found by comparing with EMBL date bank. ORF$_1$ coding for 190 amino acids including 27 arginine showed high homology with lactose trasferase of *Agrobacterium radiobacter*. ORF$_2$ coding for 255 amino acids showed 53-54% homology with *hup* E, O of *Rhizobium leguminosarum* and related with glycosylation transfer protein of *R. meliloti*.

SECTION 6

PLANT RESPONSES IN SYMBIOSIS

PR PROTEINS ARE DIFFERENTIALLY EXPRESSED DURING SYMBIOSIS

LEGOCKI, A.B.
Institute of Bioorganic Chemistry, Polish Academy of Sciences,
Poznan, Poland

Most of the attempts towards understanding the phenomenon of development and functioning of nitrogen-fixing nodules is focused on identification and characterization of the host plant genes related specifically to symbiosis. Characterization of these genes involves the time of their expression during symbiotic interactions as well as cytochemical localization of the expression products (Hirsch, 1992).

It has recently been suggested that many aspects of legume root nodulation can be explained by a simple model based on the known plant defense reactions (Mellor, Collinge, 1995). According to this model certain elements of the molecular recognition mechanisms may be common to both symbiotic and pathogenic modes of interactions.

Upon interaction with rhizobia, leguminous plants express a set of symbiosis-specific nodulin genes which participate in the nodule morphogenesis. Simultaneously, it may be important for the development of functional symbiosis to downregulate certain components of a typical host defense mechanism. A comparison of expression patterns of plant genes that are characteristic of pre-symbiotic and symbiotic stages indicates that this indeed may be the case. Recently, Sikorski et al. followed the course of nodule development in yellow lupin on the molecular level using probes for leghemoglobins - as markers of the symbiotic stage and probes for root proteins R18 - as markers of the pre-symbiotic stage. Proteins R18A and R18B representing small, acidic polypeptides of M_r 18000 are constitutively expressed in lupin roots and in other organs. Their expression is significantly reduced in organ-specific manner during nodule developement while at the same time the expression of lb genes is induced (Fig.1). A sequence analysis of the cDNA clones coding for two R18 proteins revealed that they were highly homologous to pathogenesis - related and stress - induced proteins (up to 86%). This indicates, that these proteins may be involved in the overall mechanism of plant defense. It was reported earlier, that especially at the early stages of symbiotic interaction, microsymbiont can be considered as a "refined parasite" (Djordjevic, et al, 1987) and plant recognizes bacteria as a pathogen. After the symbiosis is functionally established and effective reduction of nitrogen in mature root nodule occurs a defense mechanism is turned off. On the other hand, in senescenced nodule (starting from 36th day after inoculation) a significant increase of expression of R18 proteins is observed. At the same time a decrease of leghemoglobin level is noted. Although the function of R18 proteins is unknown they are likely to represent important, down - regulated components of the functional symbiotic system.

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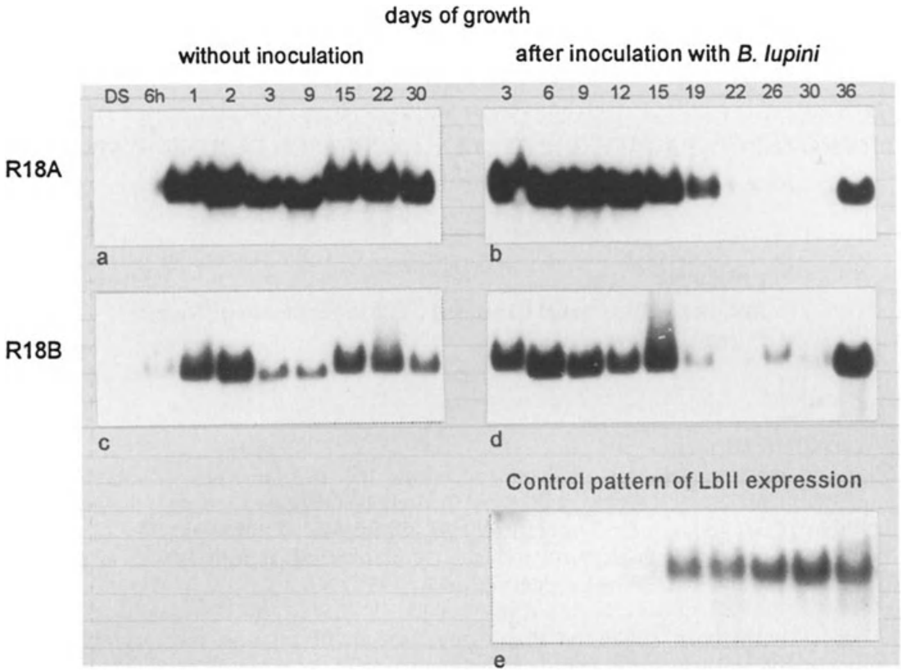
days of growth
without inoculation
after inoculation with *B. lupini*
DS 6h 1 2 3 9 15 22 30
3 6 9 12 15 19 22 26 30 36
R18A
R18B
a
b
c
d
Control pattern of LbII expression
e

Fig. 1. Expression of *L. luteus* proteins LlR18A and LlR18B during symbiosis (northern blots). Total lupin RNA were extracted from dry seeds, root of uninfected plants (a,c), infection zones and nodule tissue of plants infected with *B. lupini* (b,d,e). Hybridizations were performed with ^{32}P-labeled 3'-end fragments of cDNA clones encoding for LlR18A (a,b) and LlR18B (c,d) proteins. As a control of effective symbiosis, the RNA blots were hybridized with cDNA encoding for lupin leghemoglobin II (e).

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Hirsch AM (1992) New Phytologist 122, 211-237
Mellor RB, Collinge DB (1995) J. Exp. Botany 46, 1-18
Sikorski MM et al in preparation

MOVING CLOSER TO THE POSITIONAL CLONING OF LEGUME NODULATION GENES

GRESSHOFF, P.M.
Plant Molecular Genetics and CLR, Institute of Agriculture, The University of Tennessee, Knoxville, TN, 37901-1071, USA

I. Background:

The analysis of developmental processes in legumes is aided by genetic and molecular genetic approaches. Of special significance are processes which do not occur in other so-called model plants [Kolchinsky et al, 1994] such as *Arabidopsis thaliana* and rice. These are the nodulation and nitrogen fixation capabilities of many legumes, and the ability to interact with mycorrhizae (apparently absent in *Arabidopsis*).

The genetic analysis of legumes has been distinctly slow, despite the strong start of Mendel with pea. Research of nodulation and nitrogen fixation was even more recent, as a review of the literature in the early 1980s revealed no more than a few natural variants in soybean, pea, alfalfa and clover. Most of these were not defined beyond a general description of inheritance, and their nodulation. The use of induced mutagenesis increased the number of symbiotically defined loci, predominantly in soybean and pea, but also in chickpea, bean and sweet clover (see Caetano-Anolles, Gresshoff, 1991; Gresshoff, 1993). Some of these mutants, especially in soybean, have been described in terms of their apparent cellular blockages; however, in no case has there been an association with a biochemical step or a cloned nodulin gene.

In view of the current absense of alternative models of mutant isolation in legumes, such as insertion mutagenesis either by *Agrobacterium* T-DNA or transposable elements, the short-term prognosis for further gene characterization seems to require positional cloning or map-based cloning techniques. While these approaches have been spectacularly successful in the isolation of resistance genes in plants [Martin et al, 1993; Bent et al, 1994; Mindrinos et al, 1994], no such success has been achieved with a nodulation or nitrogen fixation controlling gene. This situation has fueled the development of the model legumes *Lotus japonicus* [Handberg, Stougaard, 1992; Jiang and Gresshoff, 1993] and *Medicago truncatula*, which are characterized by small diploid genomes and high transformation abilities, offering the possibility of insertional mutagenesis.

II. What is positional or map-based cloning?

Often mutant phenotypes cannot be associated with a protein or mRNA change. In those cases, cloning by phenotype is a viable approach. The overall strategy requires the

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association of a mutant locus with a molecular polymorphism. Originally, RFLP markers were used for this purpose, although recently methods were developed to increase the frequency of polymorphism. These newer methods are PCR-based. They include the arbitrary primer technology approaches such as DAF, RAPD and AP-PCR, the selected restriction fragment amplification (SRFA) method, also called AFLP, and microsatellites. All have been applied to enrich molecular linkage maps [Prabhu and Gresshoff, 1994]. The increased frequency of markers has been coupled with new selection strategies. At first researchers used F2 families to search for association and linkage. Now two alternatives are available. One employs near-isogenic lines (NILs), produced by repeated backcrosses to one parent, leading to a large scale genome homogenization, except for the chromosomal region surrounding the gene of interest. Accordingly, any molecular polymorphism in this region will be detected, provided sufficient numbers of probes and primers are used.

The second method is called bulk segregant analysis [BSA; Michelmore et al, 1991]. In BSA plants of a large segregating F2 are pooled according to the investigated phenotype. At times F3 plants are needed to ascertain true homozygotes. DNA from about 7 to 20 plants is pooled. Because of independent segregation and recombination, most genomic regions are equally represented in both pools. Only the chromosomal region of the investigated gene will differ, allowing the detection of molecular polymorphisms. Any detected difference needs to be checked for recombination in individuals. Any marker produced by amplification can be cloned and sequenced, then used either as an RFLP, or converted into a PCR primer pair, so that a polymorphic PCR product can be detected for the region (called a SCAR; sequence characterized amplified region). The advantage of BSA and NIL-screening with high frequency marker technology lies in the fact that there is no direct need for a map and that the technology is basically independent of genome size. Rather than spending a large effort on constructing a map and saturating a region close to a gene of interest, these approaches shift the effort to the screening of few DNA samples (two!) with perhaps 1000 functional DAF or RAPD primers.

The ultimate goal is to isolate two flanking markers within less than one centimorgan (percent recombination). Marker order is established by using recombination events in the region. This presents a paradox as the interval by definition is small, hence large populations need to be screened. This is best done by screening regional recombination events using polymorphic markers outside the mapped interval. If morphological traits are available, the search is helped. If not, molecular polymorphisms need to be used. For example, with the *nts-1* locus of soybean, we sequenced RFLP clone pA-381 and pA-36 to provide PCR primers for a PCR test of recombinants.

Once molecular analysis of just a few recombinants, or fast neutron induced deletion mutants, is achieved, the next task is the isolation of a large DNA molecule spanning the two flanking markers. If the distance is too large, several overlapping molecules will be needed. Such a contig produces an additional degree of difficulty, because plant DNA contains large amounts of repeated DNA, which would diverge a search for a neighboring DNA molecule to several genomic places. Hence, the need for a single step isolation is increased, in turn forcing the need for even closer markers.

Such large DNA molecules can be cloned into yeast artificial chromosomes (YACs). Sizes of up to a 1 megabase have been reported. YACs harbor a potential problem, as yeast is highly recombinogenic and rearrangements of the insert DNA may occur. Many YACs are chimeric. To overcome this problem, BACs, based on the F factor of *E. coli*, have been built. BACs promise insert stability, but may have smaller inserts, requiring larger collections to facilitate genome coverage.

Once a "candidate" YAC or BAC is isolated, the search for a candidate gene continues. cDNA libraries need to be screened to detect clones with homology. Any likely clone will need to be isolated from both mutant and wild-type, sequenced and compared for the likelihood of the mutant alteration in the DNA. If one cDNA is absent, analysis of genomic sequences in the cDNA region is needed. Any potential clone will then be confirmed for its coding ability through transient or stable transformation of the wild-type (presumed dominant) clone into a mutant background. If functional complementation is achieved, one can celebrate.

III. Where are we in legumes?

Research for soybean nodulation mutants focused on supernodulation and non-nodulation mutants [Gresshoff, 1993]. Two allelic EMS-induced supernodulation alleles at the *nts-1* (nitrate tolerant symbiotic) locus were mapped within about 1 cM to the pA-132 marker of the USDA/ARS RFLP map [Landau-Ellis et al, 1991]. Specifically, the closely linked marker was defined as plasmid pUTG-132a, as the originally mapped pA-132 probe was shown to contain three non-contiguous *Pst*I genomic fragments. Each detected polymorphic regions, but not in the same map region [Landau-Ellis, Gresshoff, 1994]. Detailed molecular analysis and sequencing of the linked probe showed that this region, and therefore the *nts-1* region, are present only once in the otherwise widely tetraploidized soybean genome [Kolchinsky et al, 1995].

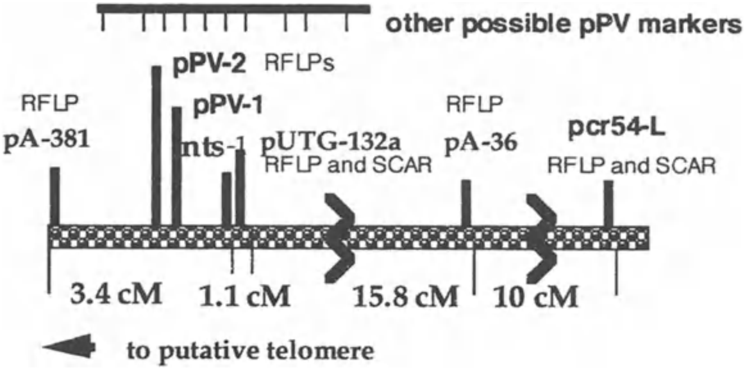

Figure 1: Molecular map in the vicinity of the *nts*-1 locus on molecular linkage group H (Iowa map). pcr54-L is a DAF generated marker converted to a SCAR (Kolchinsky et al, 1995). pPV-1 and 2 are random genomic clones from *P. vulgaris* mapped by the Shoemaker laboratory between pA-381 and pA-132. About 10 additional markers stem from this *P. vulgaris* region (Villajos E., Gainesville, FL) and are presently being mapped on the Knoxville population.

The present status of the genomic research around the *nts-1* region is summarized in Figure 1. Several candidate clones may enrich the *nts-1* region on linkage group H, if mapping results from other populations can be transferred a segregating population of *G. max* (nts382) and *G. soja* (wild-type). Fluorescent *in situ* hybridization (FISH) of the pUTG-132a probe to soybean metaphase chromosomes revealed a single chromosomal location (Paul Keim, University of Northern Arizona, Flagstaff, unpublished).

Of particular interest is the possible linkage of a histone H3 gene close to pA-132 (R.Shoemaker, pers. comm.), and the possible placement of a dozen clustered RFLP clones from *P. vulgaris* (A.Filatov, P.Gresshoff, unpubl. data, and E.Villajos, pers. comm.) in the pA-132 region. Kolchinsky et al [1995] used known homozygotes in the pA-36 to pA-381 interval of linkage group H which differed in their supernodulation phenotype to find marker pcr54L.

Using physical mapping [Funke et al, 1993] several conclusions were reached; a) markers separated by one centimorgan are contained on a fragment of about 500 kb; b) the genome is duplicated significantly, maintaining long range cutting sites, perhaps suggesting "intergenomic synteny"; and c) two-dimensional electrophoresis can resolve problems associated with genome duplication.

IV. YAC and BAC cloning of legume DNA:

HMW-DNA, isolated from agarose-embedded and lysed soybean mesophyll protoplasts, was partially digested with *Eco*RI to produce a HMW smear on PFGE. The digested DNA was size-selected using a second PFGE to eliminate molecules smaller than 250 kb. The high molecular weight DNA was isolated from the gel by melting and ligated into vector pYAC4, transformed into yeast host, and selected by colony color and uracil auxotrophy [Funke et al, 1994]. Candidate colonies were examined by PFGE and ethidium bromide staining for additional chromosomes. PFGE gels were blotted and probed with vector sequences as well as total genomic DNA. YACs in a size range of 50 to 900 kb were obtained and stored in arrayed multi-titer plates. About 7% of all clones contained chloroplast DNA. Repeated subculture, storage and regrowth detected YAC insert stability. However, the original nature of the YACs in terms of chimerism, has not ben verified.

Hybridization intensities varied for different YACs. All YACs gave equivalent signals, when probed with vector. However, only about half gave a strong signal with the total soybean DNA probe. This discrepancy is explained by the abundance of repeated DNA sequences in the genome. Intensively hybridizing YACs were presumed to harbor repeated DNA.

In this context it is relevant to indicate that soybean is known to contain from 35 to 50% repeated DNA sequences. Recently, Kolchinsky and Gresshoff [1995] isolated and sequenced a 92 bp soybean satellite DNA (SB92), which represents about 7 Mb of DNA.

The collection of soybean YACs is growing. At present about 15% of the genome is arrayed in micro-titer plates, ready for PCR screening of candidate clones. The average insert size is 200 kb, with the largest so far being found at 900 bp. As yet no clone homologous to PCR primers derived from the pUTG-132a sequence, has been found.

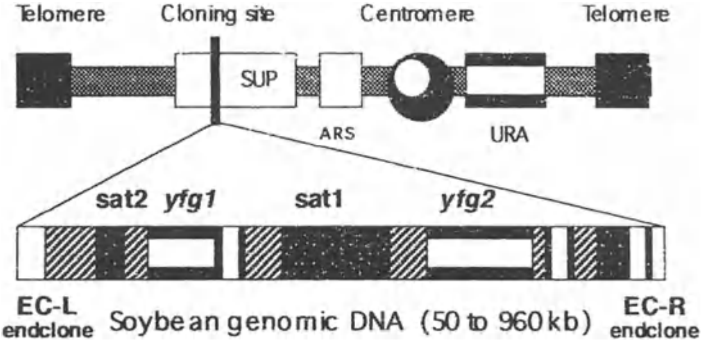

Figure 2: Diagram of soybean YAC clone. *yfg* = your favorite gene; sat = repeated DNA unit; EC = endclone (right to left). Hatched regions may harbor additional molecular markers possibly identified by arbitrary primer technology (used as DAF or RAPD; Caetano-Anolles et al, 1991) or microsatellites. Such markers, especially if they represent unique sequences, allow the detection of an overlapping YAC or BAC clone.

YAC clones are reported to suffer from chimerism. This, in part, may be caused by the highly recombinogenic yeast and the presence of repeated DNA in soybean DNA, or physical factors during transformation and spheroplast recovery. Any genome analysis project based on YACs needs to be cognoscent of the chimeric nature of used YACs, which can be detected by a variety of marker based technologies. One approach is to isolate endclones from the insert, then map these to detect cosegregation. While this approach is workable, it requires a lot of work and time. However, it generates an additional measure to evaluate regional differences in recombination frequencies and could permit the determination of some associated genomic characteristics, which influence such frequencies. Such work is especially valuable, if YACs are anchored on the molecular map through an RFLP, microsatellite or SCAR marker. Vectorette PCR allowed the isolation of insert DNA endclones from soybean YACs. Endclones hybridized to restricted DNA from *G. max* and *G. soja*, showing either repeated or unique patterns at an equal frequency. There was no prediction possible from the endclone sequence as to its repetition status. Neither was the genomic complexity of the YAC indicative of the nature of the endclone, suggesting that repeated sequences are long and interspersed. Mapping of polymorphic endclones is in progress. Figure 2 gives a diagrammatic representation of a YAC containing *yfg1* and *yfg2*, as well as dispersed repeated sequence and endclones.

Similar methods were successfull for the isolation of the first YACs from the model legume *Lotus japonicus* (S.Pillai and R.Funke, unpubl. data). This legume may offer several experimental advantages over soybean and pea. It is easily transformed by *A.tumefaciens* and *A.rhizogenes*. Regeneration from *A.tumefaciens* transformed tissue was reported, permitting the generation of many transgenic plants, perhaps opening the possibility of insertional T-DNA mutagenesis.

To develop an alternative to the likely chimeric YAC cloning system, BACs (bacterial artificial chromosomes) were constructed with both *Lotus japonicus* or soybean DNA. Here, the bacterial F factor is used to clone size-selected PFGE fragments into *E.coli.*

Presumably, recombination and chimerism will be reduced. Average insert sizes were smaller than those in YACs.

IV. Chromosome identification with YAC clones:

An additional utility of YACs is their size, facilitating the delivery of much label to a hybridization site. In collaboration with Paul Keim, YACs of different genomic complexity were used for fluorescent *in situ* hybridization (FISH) to soybean metaphase chromosomes.

YACs, especially those mapped to the molecular linkage map, can be used to identify chromosomes in trisomics. The same approach also gives strong indication concerning the chimeric nature of a YAC. YACs of differing complexities were used as templates for degenerate primer PCR. The products were labeled fluorescently and used for hybridization probes on metaphases. If a YAC belonged to the "unique" DNA class, it lit up two chromosomal regions. YACs, known to contain repeated DNA, gave multiple signals over the entire karyotype. The signal from a single YAC was 10 to 100 times stronger than that observed using the same material and equipment for a plasmid or phage clone. This procedure allows a quick determination of the chimeric nature of the cloned insert, and it facilitates a convenient way to place molecular linkage groups onto cytologically detected chromosomes.

IV. Acknowledgements:

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MAP BASED CLONING SYSTEM IN *MADICAGO* SUITABLE FOR ISOLATING GENES INVOLVED IN LEAF MORPHOGENESIS, NODULE FORMATION AND EFFECTIVENESS OF NITROGEN FIXATION

KISS G.B., KALÓ P., CSANÁDI G., FÖLFÖLDI K., KISS, P., ENDRE G.
Institute of Genetics, Biological Research Center of the Hungarian Academy of Sciences, Szeged, P. O. Box 521, H-6701, Hungary

1. Introduction

In higher eukaryotic plants, the number of the genes is approximately 50,000, therefore the plant genomes are fairly complex, consequently it is very hard to correlate genes with functions of an appropriate biological process, like symbiotic nitrogen fixation. Some techniques which are applicable for prokaryotic gene isolation (e.g. complementation of mutants) can not be used for most of the plant species. In order to correlate functions with cloned genes indirect biochemical approaches have been developed, like differential hybridization, construction of subtraction libraries, etc. Another laborious biochemical possibility is to identify a gene by its protein product. This approach involves the identification, purification and sequencing of a protein followed by the synthesis of specific oligonucleotides to use as hybridization probe or as primers for PCR. Fortunately there is an additional possibility by which specific genes can be identified: this is the genetic approach. In this case a mutant plant impaired in the appropriate biological process has to be isolated, then the gene suffered the mutation has to be genetically mapped using molecular markers, and finally the gene has to be cloned with the help of tightly linked molecular markers. This approach is the so-called map-based cloning strategy. Though the genetic strategy can not be used for every cases, nevertheless in those ones where applicable it provides an elegant solution to the problem. It has a unique advantage over the ambiguous biochemical strategies, that is one can be sure that the isolated gene is really involved and necessary in the biological process in question (see Csanádi et al., 1994).

Taking the map-based cloning strategy the first condition which has to be fulfilled is to have a mutant plant defective in the appropriate function. Mutants can be identified in the natural populations as spontaneous mutants, or mutations can be induced by chemical agents or by ionizing radiation. Mutant individuals impaired in single copy genes can be easily isolated if the mutation has no effect on the viability of the plant.

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However, it is very hard to isolate mutants if the trait is encoded by two or more gene copies, or the mutation has a deleterious effect on viability.

The mutant isolation process is followed by the genetic mapping of the mutant allele. Recently the genetic mapping is carried out by the use of molecular markers like RFLP hybridization and PCR based amplifications. The closest molecular markers can be used as start points for chromosomal walking to reach the gene of interest. Depending on the distance between the start point and the target gene, chromosomal walking can be very difficult, or relatively easy if the distance is less than 100 kilobase pairs. Nowadays map-based cloning strategy is coming into general use. This approach has been applied to clone the gene responsible for the human cystic fibrosis disease and several plant genes involved in morphogenesis or plant pathogen interactions.

2. Scientific background

Our research group has started the construction of the genetic map of alfalfa several years ago to develop a genetic system for *Medicago sativa* which can be used for basic as well as for applied research. The existence of a genetic map of alfalfa enables that genetic techniques can be applied for breeding or map based cloning type of work. In the last four years we have constructed a genetic map for alfalfa consisting of eight linkage groups using morphological, izosyme, seed protein, RFLP and RAPD markers. During our work we have crossed two diploid alfalfa plants (the yellow flowered diploid *Mecicago sativa* ssp. *quasifalcata* k93 and the purple flowered *Mecicago sativa* ssp. *coerulea* w2) which belong to the *Medicago sativa* complex (Quiros, Bauchan, 1988). One F1 plant (F1/1) was selected and self pollinated to produce the F2 population which consisted of 138 individuals. Mapping was carried out on this population by determining the genotype of more than 600 genetic markers for the individual plants in the mapping population. Among others we determined the map location of 20 nodulin genes like ENOD2, ENOD12, Nod-22, Nod-25, leghemoglobins, glutamine synthetases, and so on, as well as the location of some morphological markers like sticky leaf, dwarf, color of the flowers, etc. (Kiss et al., 1993; Kiss et al., 1994). Out of 182 genetic loci 124, 16 and 8 loci coded for single, double and triple genes, respectively. Since the duplicated regions showed no linkage, they can be explained by multiplication of smaller regions rather than duplication of larger DNA segments.

One extra-product of this work was the identification of plants which carried the ENOD12 null allele in homozygous configuration, that is no ENOD12 gene sequences were present in these plants. These plants were not affected in symbiotic nitrogen fixation consequently firm conclusion could be made, that is ENOD12 is not required for symbiosis at least under those conditions we have tested (Csanádi et al., 1994).

The long term goal of our continuous mapping effort is the isolation of genes involved in symbiotic function, that is the application of the map-based gene cloning strategy. One important prerequisite for this work is the isolation of a mutant plant impaired in the appropriate function. In our group three different mutants are under intensive investigation. The first is a leaf morphological mutant (sticky leaf), the second is a mutant exhibiting ineffective modulation, the third is a non-nodulating mutant.

3. High resolution mapping of the sticky leaf (*stl*) locus

The sticky leaf mutants are affected in leaf morphogenesis appeared in the mapping population used to construct the genetic map of alfalfa. Since its map position was determined we decided to clone the *stl* gene by map-based cloning. To reduce the number of the walking steps, molecular markers in very tight linkage with the mutation are required, that is "mapping rather than walking" is recommended.

As a first step *stl* mutation was mapped to linkage group 6 on the genetic map between RFLP markers UO553 and TMS3. These molecular markers are locating in 3.1 cM and 14.1 cM distance from *stl*, respectively, which means that they are too far to start chromosomal walking. With the application of Bulked Segregant Analysis technique (Michelmore et al., 1991) we screened RAPD primers and looked for tightly linked markers to the *stl* mutation. As it can be seen on Figure 1 we have got 36 RAPD markers in the vicinity of *stl*. In the meantime other molecular markers were also mapped close to the *stl* locus. The rRNA locus for which chromosomal location was established by Bachan and coworkers was found among them. The three tightly linked molecular markers: rRNA and the RAPDs OPP19C, OPH18A - showing no recombination with *stl* and each others in 200 segregating plants examined. 250 additional F2 plants will be further analyzed to determine the order and genetic distance between these markers.

4. Characterization and genetic analyses of the in_6 mutant of alfalfa

During the development of the alfalfa genetic system one of our aims was to isolate mutant plants impaired in symbiotic nitrogen fixation. Among the mutants isolated, one proved to be appropriate for further genetic analysis. This mutant plant was called McIN6 and the locus mutated was designated in_6. The McIN6 individual plant derived from the *Medicago sativa* ssp. *coerulea* seed population is incapable to utilize atmospheric nitrogen in the presence of *Rhizobium meliloti* AK631. The mutant plant shows the following phenotypic properties:

(i) in nitrogen free medium and in the presence of *Rhizobium meliloti* AK631 the plant displays the obvious nitrogen deficiency syndrome (retarded growth, no flower development, yellow leaf color)

(ii) in the presence of combined nitrogen (e.g. soil) this plant is able to grow normally, develops flowers and yields seeds after either self or foreign pollination.

(iii) the ineffective phenotype is maintained after either vegetative or generative propagation.

(iv) in the absence of combined nitrogen but in the presence of *Rhizobium meliloti* AK631 many white (leghemoglobin minus), ineffective nodules are formed on the roots;

the number of the nodules could be 10-20 times more compared to the wild type nodule number.

(v) light microscopical analysis showed that the bacteria enter the nodule cells and they develop into bacteroids but inside the nodules few infected cells are found; the invasion process takes place normally (infection threads are visible), though the number of the bacteroids is far less than in the normal nodules.

(vi) electron-microscopical analysis revealed that the nodule cells undergo early senescence; this phenomenon is similar to the *in*$_2$ and *in*$_3$ mutants isolated from tetraploid alfalfa cultivar (Vance, Johnson, 1983)

(vii) while homozygote mutant plants are not able to form effective symbiosis with strain AK631 (an Exo$^-$ derivative of *Rhizobium meliloti* 41), it seems that they do form some nitrogen fixing nodules with strain RM41 which is Exo$^+$ (Putnoky et al., 1990). Both strains establish effective symbiosis with wild type alfalfa plants.

In order to find out the inheritance of the *in*$_6$ mutation genetic crosses were performed. From the cross between McIN6 and the wild type Mcw2 plant it was clear that the mutation is recessive, since all F1 progenies were able to fix nitrogen. Self mating progeny of the F1 plants showed that the mutation inherited as a single Mendelian marker.

The McIN6 plant derived from *Medicago sativa* ssp. *coerulea* had been also crossed with the yellow flowered *Medicago sativa* ssp. *quasifalcata* k93 plant and F1 as well as F2 (S1/F1) plants were generated. 29 ineffective and 180 effective F2 plants from self mating of two F1 plants (F1/19 and F1/51) were selected for genetic mapping of the *in*$_6$ mutation. The F2 progenies to be analyzed were planted into pots and grown in the greenhouse. RFLP (Restriction Fragment Length Polymorphism) and RAPD (Random Amplified Polymorphic DNA) markers were used to map the *in*$_6$ mutation on tne eight linkage groups of the genetic map of *Medicago* (Kiss et al., 1993). 16 and 24 selected known RFLP and RAPD markers, respectively, were used to determine the genotype of the appropriate loci for the ineffective plants. These markers covered more than 70 percent of genome in 20 cM intervals.

Simultaneously we also tried to find linkage of *in*$_6$ mutation to such molecular markers for which the map position has not yet been determined. One RAPD marker (OPE7X) showed linkage with the *in*$_6$ allele. Mapping of the OPE7X marker on the mapping population it turned out that it is located on linkage group 7 between markers UO69 and L83 (see Figure 2).

The diploid *Medicago sativa* ssp. *coerulea* IN6 (McIN6) mutant plant of alfalfa carrying the *in*$_6$ mutation is responsible for ineffective nodulation and fulfills conditions to start map based cloning experiments. This work involves (i) the fine mapping of the *in*$_6$ allele within 0.5 centimorgan distance, (ii) determination of the physical distance between the closest molecular markers bordering the mutated gene, (iii) the construction of a genomic library from a plant with the appropriate genotype, (iv) the chromosomal walk by the isolation of overlapping clones, (v) and start the complementation of the mutation by transformation using the isolated overlapping clones. In parallel experiments plant nodulation test with different *Rhizobium meliloti* strains is performed, the expression of the different nodulin genes is determined in the nodules of the in_6 plants, and plant defense reactions is tested to try to understand the biological lesion leading to the ineffective phenotype.

5. Generating diploid non-nodulating alfalfa plant from a tetraploid germplasm

The tetraploid MnNC-1008(NN) germplasm is a non-nodulating mutants of alfalfa (Peterson and Barnes, 1981). This plant is unable to form symbiotic nodules, there is no root hair curling nor cortical cell division after rhizobial infection (Dudley and Long, 1989). This ensures that the recognition or signal transduction process eliciting by the first bacterial signals is affected, that is a very early stage in the nodule organogenesis is impaired in this mutant, consequently the wild type alleles of the affected genes must have indispensable function in the nodulation process. Since the inheritance of two unlinked genes, *nn1* and *nn2* in a tetraploid segregating population is excessively complicated we decided to reduce the ploidity of MnNC-1008(NN) to diploid level. Following the procedure described by E. T. Bingham (1969) one diploid plant was generated after more than 7000 cross-pollination. The details of this procedure is described by Endre and coworkers in this Proceedings.

6. Conclusion

The saturated linkage map of alfalfa having eight linkage groups and more than 600 markers allows us the mapping of mutations affecting different traits of *Medicago sativa* like leaf morphology, nodule development and symbiotic effectiveness. Closely linked molecular markers (within 0.5 centimorgan) can be used as starting points for chromosomal walking experiments. This map-based cloning strategy will enable us to identify and characterize genes required for leaf development, nodule organogenesis and symbiotic function of the root nodules in alfalfa.

7. Acknowledgment

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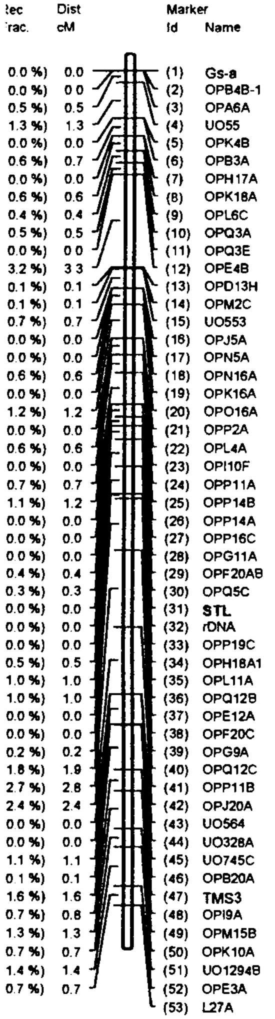
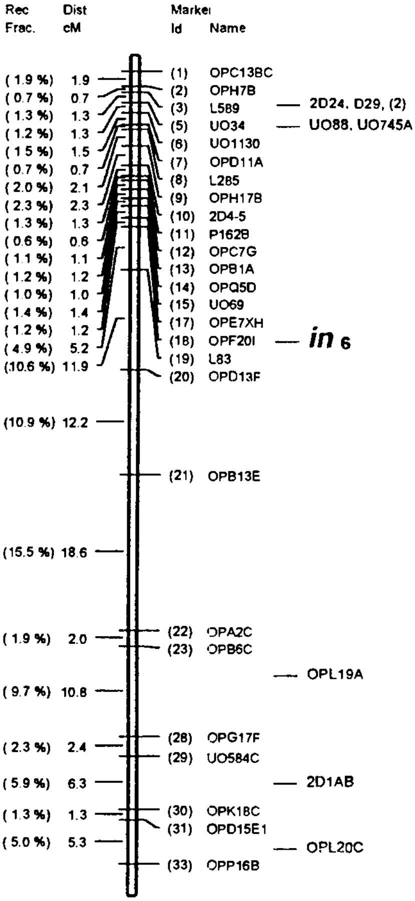

ec rac. | Dist cM | Marker Id Name
0.0 %) 0.0 (1) Gs-a
0.0 %) 0.0 (2) OPB4B-1
0.5 %) 0.5 (3) OPA6A
1.3 %) 1.3 (4) UO55
0.0 %) 0.0 (5) OPK4B
0.6 %) 0.7 (6) OPB3A
0.0 %) 0.0 (7) OPH17A
0.6 %) 0.6 (8) OPK18A
0.4 %) 0.4 (9) OPL6C
0.5 %) 0.5 (10) OPQ3A
0.0 %) 0.0 (11) OPQ3E
3.2 %) 3.3 (12) OPE4B
0.1 %) 0.1 (13) OPD13H
0.1 %) 0.1 (14) OPM2C
0.7 %) 0.7 (15) UO553
0.0 %) 0.0 (16) OPJ5A
0.0 %) 0.0 (17) OPN5A
0.6 %) 0.6 (18) OPN16A
0.0 %) 0.0 (19) OPK16A
1.2 %) 1.2 (20) OPO16A
0.0 %) 0.0 (21) OPP2A
0.6 %) 0.6 (22) OPL4A
0.0 %) 0.0 (23) OPI10F
0.7 %) 0.7 (24) OPP11A
1.1 %) 1.2 (25) OPP14B
0.0 %) 0.0 (26) OPP14A
0.0 %) 0.0 (27) OPP16C
0.0 %) 0.0 (28) OPG11A
0.4 %) 0.4 (29) OPF20AB
0.3 %) 0.3 (30) OPQ5C
0.0 %) 0.0 (31) STL
0.0 %) 0.0 (32) rDNA
0.0 %) 0.0 (33) OPP19C
0.5 %) 0.5 (34) OPH18A1
1.0 %) 1.0 (35) OPL11A
1.0 %) 1.0 (36) OPQ12B
0.0 %) 0.0 (37) OPE12A
0.0 %) 0.0 (38) OPF20C
0.2 %) 0.2 (39) OPG9A
1.8 %) 1.9 (40) OPQ12C
2.7 %) 2.8 (41) OPP11B
2.4 %) 2.4 (42) OPJ20A
0.0 %) 0.0 (43) UO564
0.0 %) 0.0 (44) UO328A
1.1 %) 1.1 (45) UO745C
0.1 %) 0.1 (46) OPB20A
1.6 %) 1.6 (47) TMS3
0.7 %) 0.8 (48) OPI9A
1.3 %) 1.3 (49) OPM15B
0.7 %) 0.7 (50) OPK10A
1.4 %) 1.4 (51) UO1294B
0.7 %) 0.7 (52) OPE3A
(53) L27A

Figure 1. Fine mapping of STL region on the Linkage Group 6 of the genetic map of alfalfa


Rec Frac. | Dist cM | Marker Id Name
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(0.7 %) 0.7 (2) OPH7B
(1.3 %) 1.3 (3) L589 — 2D24, D29, (2)
(1.2 %) 1.3 (5) UO34 — UO88, UO745A
(1.5 %) 1.5 (6) UO1130
(0.7 %) 0.7 (7) OPD11A
(2.0 %) 2.1 (8) L285
(2.3 %) 2.3 (9) OPH17B
(1.3 %) 1.3 (10) 2D4-5
(0.6 %) 0.6 (11) P162B
(1.1 %) 1.1 (12) OPC7G
(1.2 %) 1.2 (13) OPB1A
(1.0 %) 1.0 (14) OPQ5D
(1.4 %) 1.4 (15) UO69
(1.2 %) 1.2 (17) OPE7XH
(4.9 %) 5.2 (18) OPF20I — in6
(10.6 %) 11.9 (19) L83
(20) OPD13F
(10.9 %) 12.2
(21) OPB13E
(15.5 %) 18.6
(1.9 %) 2.0 (22) OPA2C
(23) OPB6C
— OPL19A
(9.7 %) 10.8
(2.3 %) 2.4 (28) OPG17F
(29) UO584C
(5.9 %) 6.3 — 2D1AB
(1.3 %) 1.3 (30) OPK18C
(31) OPD15E1
(5.0 %) 5.3 — OPL20C
(33) OPP16B

Figure 2. Map position of *in6* mutation on the Linkage Group 7 of the genetic map of alfalfa

ROLE OF PLANT HORMONES AND CARBON/NITROGEN METABOLISM IN CONTROLLING NODULE INITIATION ON ALFALFA ROOTS

BAUER P.[1], COBA DE LA PENA T.[1], FRUGIER F.[1], POIRIER S.[1], MCKHANN H.I.[1], RATET P.[1], BROWN S.[1], CRESPI M.[1], KONDOROSI A.[1,2]
[1]Institut des Sciences Végétales, CNRS, F-91198 Gif sur Yvette, France
[2] Institute of Genetics, Biological Research Center, Hung. Acad. of Sciences, H-6701 Szeged, Hungary

INTRODUCTION

Interactions between rhizobia and their leguminous plant hosts result in the formation of nitrogen-fixing root nodules. All steps of nodule development are controlled by the plant. To induce nodule formation and its invasion bacteria produce host-specific lipo-chitooligosaccharide signal molecules, the Nod factors. Purified Nod signals trigger various plant responses (Schultze et al., 1994). It has been suggested that separate signal transduction pathways may exist for inducing events related to the invasion process, such as root hair deformations and formation of preinfection threads, and for eliciting cortical cell divisions leading to nodule development (Ardourel et al., 1994). Interestingly, only the latter ones could be mimicked by the exogenous supply of plant hormones or auxin transport inhibitors, suggesting that pathways of plant hormones might be exploited at least partly by the Nod factors (Hirsch et al., 1994).

We have been studying the early plant responses induced by the *R. meliloti* Nod signals in *Medicago* aiming at the understanding of the molecular events involved in nodule primordium formation. These include identification of *Medicago* genes and pathways induced or enhanced by the *R. meliloti* Nod factors.

Recently, we reported that *M. sativa* harbors two *Enod12* genes, that are differentially expressed during nodule development (Allison et al., 1993; Bauer et al., 1994). Both genes encode putative proline-rich cell wall proteins. *MsEnod12B* expression patterns resembled the activation of *Enod12* genes identified in other legumes (Scheres et al.,1990; Pichon et al., 1992; Horvath et al., 1993; Journet et al., 1994). Using reverse transcription-PCR, its inducton was detected within six hours after inoculation with *R. meliloti* and treatment with the purified NodRmIV(C16:2,S). In contrast, *MsEnod12A* transcripts were detected only when nodules became visible (Bauer et al., 1994). Moreover, we have identified two cDNA clones isolated by differential screening of a young nodule cDNA library from *M. sativa* using RNA isolated from roots and from spontaneous nodules, developed in the absence of bacteria. One of these cDNAs turned to be the homolog of the the soybean *GmEnod40* genes (Yang et al., 1993; Kouchi, Hata,1993)and was named *MsEnod40* (Crespi et al., 1994). The other cDNA showed strong sequence similarity to carbonic anhydrase genes expressed in leaves (Burnell and Hatch, 1988, Hatch and Burnell, 1990).

Here, we report the use of these three genes as molecular markers for studying the early events of nodule development. Using *in situ* hybridization the temporal and spatial

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expression patterns of these genes have been analyzed during nodule organogenesis. In addition, transgenic alfalfa plants carrying the *MsEnod12A* promoter-GUS fusion were generated, and then used for studies on the possible role of plant hormones and the plant carbon/nitrogen metabolism in the control of the onset of cortical cell divisions.

MsEnod12A is a marker gene for the onset of root and nodule organogenetic programs

The spatial and temporal expression of *MsEnod12A* was followed during nodulation by *R. meliloti*, using transgenic *M. sativa* ssp.*varia* A2 plants containing the *MsEnod12A*-GUS fusion. After inoculation with *R. meliloti MsEnod12A* was first induced in the dividing cortical cells, whereas no GUS activity was detected in the root epidermis, in deformed or curled root hairs as described for other *Enod12* (Scheres et al., 1990; Pichon et al., 1992; Horvath et al.,1993; Journet et al., 1994). In mature nodules expression was restricted to the site near the meristem, as also shown by *in situ* hybridization (Bauer et al., 1994). After application of purified Nod factors (10-6M NodRm-IV(C16:2,S)) gene expression was localized to the dividing cortical cells. Again no GUS staining was found in the root hairs or the epidermis during the first days of treatment. Similar expression patterns were detected during nodulation with different *Rhizobium* mutant strains and also in spontaneous nodules, further supporting that *MsEnod12A* expression was related to nodule organogenesis. Interestingly, we found GUS staining also during lateral root formation, albeit at a much lower intensity, at the initiation sites of lateral and adventitious roots and behind the root apices.

These data indicate that *MsEnod12A* expression occurs during the development of a primordium and is maintained after the generation of a discrete meristem, both of nodules and lateral roots, suggesting that the factors operating on *MsEnod12A* transcription are not exclusively involved in nodulation. The higher GUS activity during nodulation can be explained by tissue specificity, requirements for anticlinal cell division planes, the plant hormonal balance and/or nitrate availability. Primordium- or meristem-related expression might indicate a possible function of proline-rich proteins to specify cell contacts inside clusters of cells destined to the same developmental pathways.

Effects of exogenously applied plant growth regulators in comparison to Nod factor action

The *MsEnod12A*-GUS fusion is expressed during nodule as well as lateral root initiation in alfalfa, and thus can be used as a tool to visualize molecular events in terms of GUS staining patterns and intensities, and to correlate them with morphological alterations observed on the roots after treatments with various growth-modulating substances. We compared the early effects of cytokinins, auxin transport inhibitors and auxin-like compounds to the action of Nod factors with regard to the following criteria: induction of cortical cell division foci where *MsEnod12A* expressed, amyloplast deposition, the susceptible regions of the root and dependence on limited supply of combined nitrogen. We found that, similarly to Nod factor NodRmIV(C16:2,S), cytokinin (10-5 and 10-6M BAP) treatment for four days could induce clusters of cortical cell divisions in the inner cortex where *MsEnod12A* expressed. In both treatments we observed starch granule formation all along the cortex of main and lateral roots. Cortical cell divisions were observed most strongly in the root hair and young lateral root zones of tap roots, and also in the lateral roots of the upper main root. The root tips hardly or did not show any GUS activity in front of the apices.

The induction of cortical cell divisions and starch accumulation by either cytokinins or Nod factor was dependent on certain environmental conditions. In the presence of nitrate as the only nitrogen source, cytokinin or Nod factor action was strongly reduced. We also found that the conditions for plant growth and treatments could influence the plant responses towards cytokinins or Nod factors.

In contrast to cytokinins, exogenously applied auxins-like compounds (10-5 and 10-6M 2,4D; 10-5M NAA) for four days had very different effects on the roots. The alterations in root morphology, starch accumulation in the inner cortical cells seemed to be basically related to the generation of lateral root bumps and statolithe formation all along the root. Very typical and strong GUS staining patterns were detected near the swollen tips of the roots. In the presence of nitrate the auxin effects were even reinforced.

Auxin transport inhibitors (10-5M NPA) showed a delayed response towards cortical cell divisions Clusters of strongly GUS-stained and dividing cortical cells were first clearly recognized after eight days. Further complex changes occurred on the plant roots, partly associated with secondary root growth. We found that auxin transport inhibitors had also activated the endodermis and the pericycle, cambium formation and vascular tissue differentiation. Starch was not accumulated in the cortex but in newly generated cells inside the former pericycle. The tips of these roots showed strong GUS activity as it was also observed after certain auxin treatments.

These studies indicate that exogenously applied cytokinins have very similar effects on the roots compared to those of Nod factors, suggesting that the early plant responses evoked by Nod factors may be linked at least partly to the cytokinin signal transduction pathways.

Carbon/nitrogen metabolism and cytokinin action

We discussed above that the changes leading to nodule initiation (anticlinal cortical cell divisions, *MsEnod12A* expression, amyloplast deposition) can be induced by *R. meliloti*, Nod factors and cytokinins only under nitrogen starvation. Accumulation of starch suggests that not only the nitrogen, but also the carbohydrate metabolism or their ratio may influence the onset of nodule development. We therefore investigated whether the elevated carbon/nitrogen ratio, as a consequence of nitrogen starvation, was implicated in the control of cytokinin action. Therefore, in our experiments photosynthesis was blocked by incubating the plants in the dark during the cytokinin treatment in the absence of nitrogen supply. This treatment almost completely abolished the cytokinin-inducibility of cortical cell divisions and starch accumulation in the cortex. Interestingly, we observed that under these conditions cytokinins still induced lateral root primordia. No primordia were observed in the absence of cytokinins. Recent observations of other groups also indicate that cytokinins have a role in lateral root formation, in contrast to previous assumptions (E. Schoonejans, pers. comm.; Hamill, 1993).

Our observations suggest that the susceptibitlity for the BAP induction of cell divisions in the cortex or in the pericycle was dependent on the metabolic state of the root. Preliminary results indicate that addition of sucrose to cytokinin-treated dark grown plants in the absence of nitrate could restore the induction of cortical cell divisions.

We therefore conclude that the induction of cortical cell divisions is controlled by metabolic intermediates or by signals derived from an altered carbon/nitrogen metabolism.

Enod40 may be involved in plant growth

After inoculation of alfalfa roots with *R. meliloti*, *MsEnod40* transcripts were first detectable in the pericycle opposite to a xylem pole and in the dividing cortical cells. Later the transcripts were localized to the differentiating nodule cells but not in the terminally differentiated ones in the symbiotic zone. Treatment of alfalfa roots with Nod factor enhanced the expression of this gene (Crespi et al., 1994). Moreover, the exogenous application of cytokinins to the roots also induced the expression of this gène (Hirsch et al., 1994).

Surprisingly, only short open reading frames (ORF) were found both in the cDNA and in the genomic *Medicago* sequences, as well as in the cDNAs of *Glycine max* (Crespi et al., 1994). However, on DNA level *Medicago and G. max Enod40* sequences were well conserved all along the transcripts. Calculating the stability of *Enod40* RNA secondary structures we found that both the alfalfa and soybean *Enod40* transcripts possess low free energies of folding comparable to the values obtained for non-coding, highly structured RNAs. These data strongly suggest that the *Enod40* gene product might be a developmentally-regulated untranslated RNA, though the translation of small existing ORFs into oligopeptides cannot be excluded. Interestingly, polyadenylated non-coding RNAs or 3'UTR regions of mRNAs have been recently identified as tumor suppressors in mammalian cells playing a role as "riboregulators" in differentiation and growth control (Rastinejad and Blau, 1993). We investigated the similar function of the *Enod40* RNA in transgenic *Medicago sativa* and observed inhibited callus growth and embryo formation in the case of the antisense construct under the control of the constitutive 35S promoter whereas overexpression of *MtEnod40* produced polyembryogenic calli that displayed poor and delayed regeneration capacity. These plant phenotypes suggest that the *Enod40* gene product may affect hormonal balances in the plant. This is supported by preliminary results obtained after overexpression of *MtEnod40* in *Arabidopsis* which led to altered growth of seedlings. We suggest that *Enod40*, apart from its role in nodule organogenesis, might be involved in other developmental processes and may possibly represent the first example of a plant riboregulator.

Carbonic anhydrase, a new early nodulin

Using *in situ* hybridization, we showed that the *M. sativa* carbonic anhydrase gene, *Msca1*, exhibited specific temporal and spatial patterns during nodule development. The gene was induced very early in all cells of the nodule primordium. After differentiation of nodules induced by wild type *R. meliloti*, its expression was restricted to the peripheral cells forming a barrier all around the nodule organ, thus displaying a novel nodulin gene expression pattern. In contrast, *Msca1* expression in empty nodules induced by *exo* mutants of *Rhizobium* as well as in spontaneous nodules was localized to the center of the nodules suggesting that the presence of rhizobia was required for the peripheral expression pattern. *Msca1* may be partly regulated by metabolic signals. Nodules elicited by *R. meliloti nif* mutants showed a similar expression patterns as wild type nodules, indicating that fixed nitrogen is not involved in its regulation. Interestingly, using transgenic plants containing a PEPc promoter-GUS fusion similar pattern was observed (C. Vance, pers. comm.). The role of CA in leaves is to provide carbonic acid to PEPC by hydration of CO2; it may have the same function in the developing as well as the symbiotic nodules. Thus, the *Msca1* gene may be involved in the carbon metabolism of the nodules and may serve as a marker for these studies.

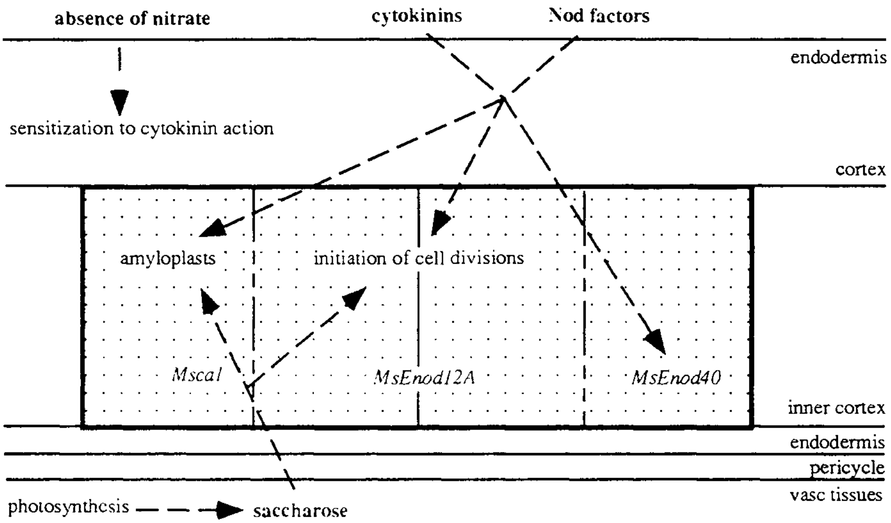
absence of nitrate
cytokinins
Nod factors
endodermis
sensitization to cytokinin action
cortex
amyloplasts
initiation of cell divisions
Mscal
MsEnod12A
MsEnod40
inner cortex
endodermis
pericycle
vasc tissues
photosynthesis
saccharose

Fig.1: Model for Nod factor/cytokinin action on nodule initiation and control by the carbon/nitrogen metabolism
A root starved for nitrogen is sketched with the beginning of anticlinal cell divisions in the inner cortex. It is not known whether Nod factors release cytokinins in the outer cortex or act on downstream elements of the cytokinin signal transduction pathway. Interrupted arrowheads indicate a series of events leading to the observed phenomena. To keep this scheme simple, further plant hormones and plant factors implicated in the maintenance and differentiation of the primordia are not included here.

CONCLUSIONS

By following *MsEnod12A* expression and morphological responses in the root after various treatments with growth-regulating compounds we found that exogenously applied Nod factors and cytokinins had very similar effects on nitrogen starved roots. In addition to the induction of pseudonodules by cytokinin-overproducing bacteria and the induction of early nodulin genes by cytokinins (Hirsch, Fang, 1994; Cooper and Long, 1994; Dehio and de Bruijn, 1992), our results provide strong evidence that Nod factors act on cytokinin-related signaling mechanisms leading to nodule initiation. Even though cytokinins may induce the initial cell divisions in the cortex and also in the pericycle, during nodule and lateral root formation the maintenance of the primordium and its differentiation are more likely to be achieved by further plant hormonal signals (Hamill, 1993; Estelle and Klee, 1994). Our results indicate that *Enod40* is able to modify the plant hormonal balance and as a plant growth modulator it may act throughout nodulation.

Fig.1 summarizes our observations on the control of cytokinin and Nod factor action by the metabolic status of the root. Preliminary results suggest that biochemical intermediates of the carbon/nitrogen metabolism are involved in this regulation. Analysis of metabolic enzymes, such as carbonic anhydrase, expressing during nodule initiation may help elucidating the nature of this (these) carbon signal(s) which may be derived separately from the carbon and nitrogen metabolisms or may represent the carbon/nitrogen ratio.

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SYMBIOSOMES UNDER CONTROL OF PLANT AND MICROSYMBIONT GENES

WERNER D, JACOBI A, WINZER T and MÜLLER P
Philipps-Universität, FB Biologie, Karl-von-Frisch-Straße, D-35032
Marburg, Germany

INTRODUCTION

Biochemistry, cell biology and molecular biology of symbiosome compartments and development have been reviewed and discussed by Werner (1992), Verma et al. (1993), Kardailsky and Brewin (1994), McKahnn and Hirsch (1994) and Schultze et al. (1994). The symbiosome membrane is a physiological barrier, preventing the symbiont from being recognized as a parasite (Werner, 1992). The membrane is involved in hormonal perception (Jacobi et al. 1993) and in transport processes from the bacteroids and to the bacteroids (Udvardi et al. 1990). From the nodulins of the symbiosome membrane nodulin 26 has a known biochemical function with the transport of dicarboxylic acid, regulated by phosphorylation (Weaver et al. 1991). The symbiosome fluid has been characterized e.g. by a protease inhibitor (Garbers et al. 1988) and in new lectine type glycoprotein (Kardailsky and Brewin, 1994).

PLANT EFFECTS ON THE SYMBIOSOME MEMBRANE PROTEINS AND NODULINS

The polypeptide composition of the symbiosome membrane in soybean root nodules is already completed 24 days after infection and remains essentially unaffected up to 73 days (Jacobi et al. 1994). Only one protein was more abundant in younger nodules and three others were more abundant in older nodules. Compared to physiological ageing, induced nodule senescence by decapitation dramatically changes the polypeptide composition of the symbiosome membrane, discriminating between membrane nodulins and the other membrane proteins. Most symbiosome membrane nodulins are not detectable five days after induced senescence, including nodulin 26. Nodulin 26 is an integral membrane protein phosphorylated by a calcium dependent, calmodulin independent nodulin kinase (Miao et al. 1992). This nodulin has a sequence homology to other tunnel proteins such as the tunnel protein in *E. coli*, transporting glycerol and to integral membrane proteins in erythrocytes and in plant tonoplasts with functions as water channels.

Two-dimensional maps of symbiosome membrane proteins by 2-D-PAGE are shown in Fig. 1. Major differences are marked with arrows in panel A and B, some polypeptides disappear completely where other are quantitatively reduced.

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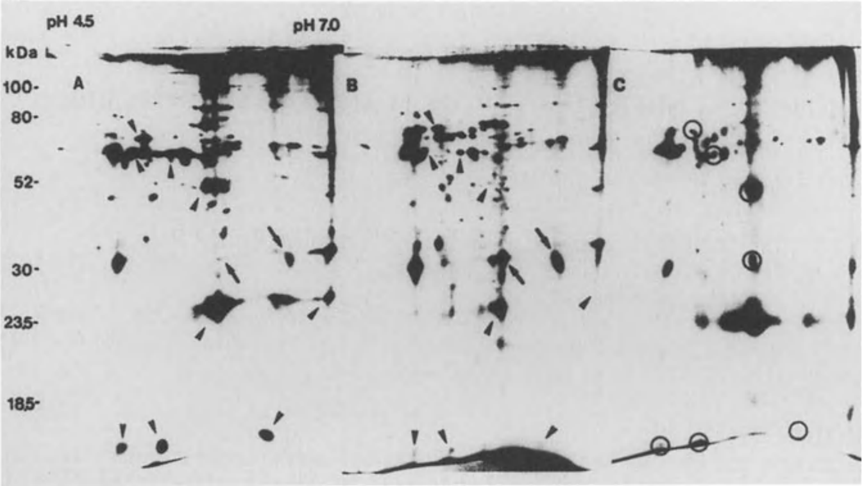


Fig. 1: Comparative analysis of SM proteins by 2-D-PAGE revealed that some proteins of *Bradyrhizobium japonicum* 184 induced nodules in *Glycine max* were less abundant than in wild-type induced nodules (panel A) as indicated by open circles in panel C. Similar results were observed with nodules of induced senescence (panel B).

MICROSYMBIONT GENES AFFECTING THE SYMBIOSOME MEMBRANE: SIGNAL PEPTIDASES

The biochemical dialogue in the interaction between rhizobia and their various legume host plants can be impaired by bacterial mutations at different steps of nodule formation. Using Tn*phoA* for random mutagenesis of *B. japonicum* 110*spc*4 led to the isolation of a series of mutants which symbiotically reduced N_2 at a lower level whereas their *ex planta* nitrogenase activity was still maintained. Two mutant strains, 132 and 184, were analyzed in more detail.

Light microscopic investigations revealed that mutant 132 infected soybean nodule cells were extensively vacuolized. Callose formation was observed in some central nodule cells, which were in contact with infection threads, indicating plant defense reactions in this advanced step of nodule development, 24 dpi. A spectroscopic characterization of the cytosolic fraction of root nodules revealed that the functional ferrous leghemoglobin was replaced by the inactive ferric form. Furthermore, immunohistochemical studies documented that the presence of PEPcarboxylase, which can be regarded as a nodulin providing carbon skeletons for ammonium assimilation, was clearly reduced. An antibody directed against proteins of the symbiosome membrane resulted in weaker signals in thin sections of mutant induced nodules. In contrast, chitinase was detected in infected tissue at higher concentrations compared to wild-type nodules. Electron microscopic studies show that cells of mutant strain 132 are stable within infection threads. As soon as they are released to the plant cytosol and thereby engulfed by the symbiosome membrane they rapidly deform and finally undergo lysis. Therefore the mutation in strain 132 has a strong influence on the protein composition of the symbiosome membrane and on the function of this membrane. At the end the "symbiosomes" become "lysosomes" which fuse and form large central vacuoles.

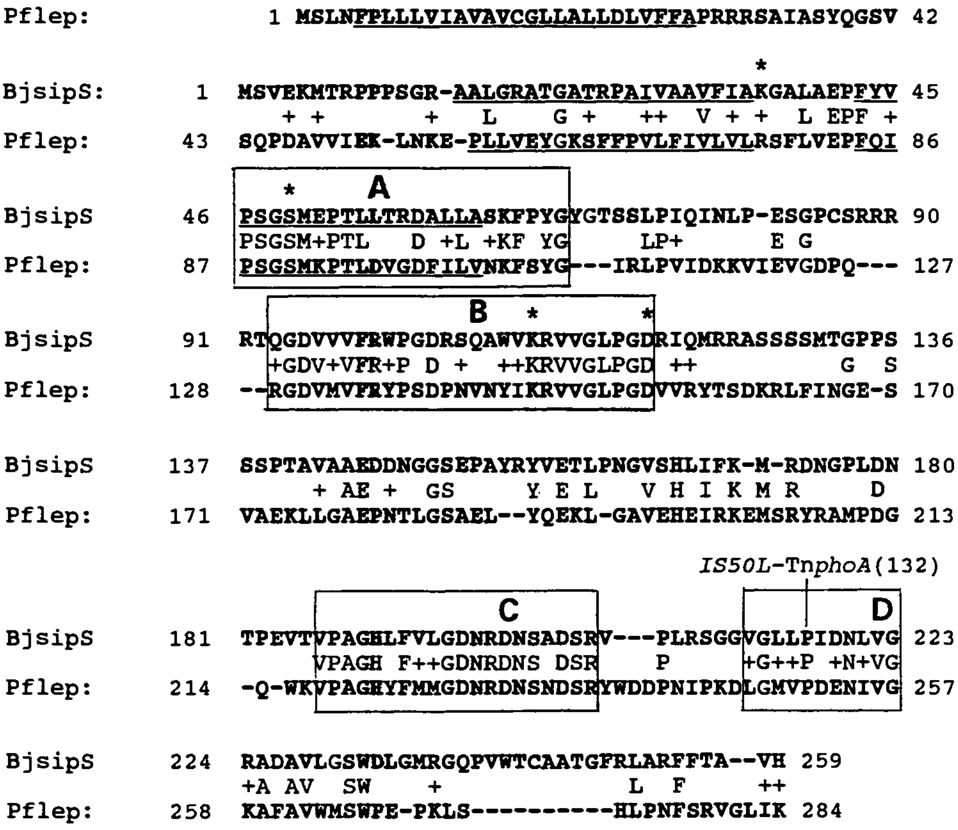
In order to learn more about the mutation in strain 132, a 4.4 kb *Bam*HI wild-type DNA fragment was cloned and the nucleotide sequence was determined. Furthermore the exact Tn*phoA* insertion site was localized and found to be in the C-terminus of ORF2. When the nucleotide sequence was compared with the datalibrary by the blastx programm, homologies to procaryotic type I signal peptidases were found. The best fit was obtained with the *lep* gene of *Pseudomonas fluorescens*, since both gene products have a similar length (284 versus 259 aa). The difference in length can be primarily explained by the lack of the N-terminal hydrophobic region of *Pflep* in the *B. japonicum* SipS protein (Fig. 2), which is also missing in signal peptidases of various *Bacillus* strains. Since the mutation in *B. japonicum* 132 resulted in a clearly detectable phenotype only under symbiotic conditions but not with free-living cells, this locus was designated *sipS*.

The homology to signal peptidases (Fig. 2) was surprising, since *lep* mutations in *E. coli* are lethal, indicating that this gene is absolutely essential for the processing of other preproteins. However, Inada et al. isolated a temperature sensitive *E. coli lep* mutant, IT41, which grows at 32°C but not at 42°C (Inada et al., 1989). Strain IT41 was used to test different *B. japonicum* subclones whether they complemented the conditional lethal mutation. Hybrid plasmids carrying DNA fragments with an intact *sipS* gene and providing a vector-borne promoter in the proper orientation complemented the mutation. Therefore *B. japonicum sipS* codes for a signal peptidase which is functional also in a heterologous microorganism. This result means that in *B. japonicum* more than one signal peptidase must exist.

B. japonicum sipS is preceeded by ORF1. Since the TGA stop codon of ORF1 and the ATG start codon of sipS overlap, a tight translational coupling can be assumed. The deduced amino acid sequence of ORF1 is highly homologous to ORF481 of *E. coli* which function remains to be determined. Downstream of *sipS* a third ORF is located which forms the last gene of this putative operon. The inactivation of ORF3 (334 aa) by an *aphII* resistance cassette shows that this gene product is not required for complementation of the *E. coli* *lep*^{ts} mutant IT41, and its inactivation does not cause a symbiotic phenotype in *B. japonicum*. Therefore a polar effect of the Tn*phoA* insertion in strain 132 can be excluded.

The Tn*phoA* insertion in mutant 132 results in the formation of a 70 kD fusion protein which can be detected in a Western analysis, using an antibody directed against the alkaline phosphatase which fits well with the estimated size based on the sequence data. In addition, a prominent protein band of 52 kD is detected, which might be a specific degradation product. When whole cells of *B. japonicum* 132 are assayed for alkaline phosphatase activity, a marked increase of specific enzyme activity can be measured with free-living cells. Thus this operon is expressed constitutively and does not require plant derived inducers.

The symbiotic phenotype of mutant 184 is characterized by an abnormal pattern of nodule colonization. The mutants infect the plant root via infection thread, but the bacteria are released only in a few cells of the central nodule tissue which leaves the majority of cells uninfected. This can be explained by the slow growth rate of mutant 184. The bacteroids of mutant 184 are surrounded by a symbiosome membrane, but they are irregularly shaped and perform symptoms of early senescence. Preliminary and so far incomplete data obtained from sequence analysis of the DNA region that has been tagged by Tn*phoA* indicate that a second type I signal peptidase is mutated in *B. japonicum* 184.



```
Pflep:           1 MSLNFPLLLVIAVAVCGLLALLDLVFFAPRRRSAIASYQGSV 42

                                                   *
BjsipS:     1  MSVEKMTRPPPSGR-AALGRATGATRPAIVAAVFIAKGALAEPFYV 45
                  + +       +   L    G +   ++  V + +  L EPF +
Pflep:     43  SQPDAVVIEK-LNKE-PLLVEYGKSFFPVLFIVLVLRSFLVEPFQI 86

                  *     A
BjsipS     46  PSGSMEPTLLTRDALLASKFPYGYGTSSLPIQINLP-ESGPCSRRR 90
               PSGSM+PTL   D +L +KF YG     LP+      E G
Pflep:     87  PSGSMKPTLDVGDFILVNKFSYG---IRLPVIDKKVIEVGDPQ--- 127

                               B   *       *
BjsipS     91  RTQGDVVVFRWPGDRSQAWVKRVVGLPGDRIQMRRASSSSMTGPPS 136
                 +GDV+VFR+P D +  ++KRVVGLPGD ++          G  S
Pflep:    128  --RGDVMVFRYPSDPNVNYIKRVVGLPGDVVRYTSDKRLFINGE-S 170

BjsipS    137  SSPTAVAAEDDNGGSEPAYRYVETLPNGVSHLIFK-M-RDNGPLDN 180
                    + AE +  GS     Y E L   V H I K M R     D
Pflep:    171  VAEKLLGAEPNTLGSAEL--YQEKL-GAVEHEIRKEMSRYRAMPDG 213

                                               IS50L-TnphoA(132)
                                 C                    |    D
BjsipS    181  TPEVTVPAGHLFVLGDNRDNSADSRV---PLRSGGVGLLPIDNLVG 223
                    VPAGH F++GDNRDNS DSR    P     +G++P +N+VG
Pflep:    214  -Q-WKVPAGHYFMMGDNRDNSNDSRYWDDPNIPKDLGMVPDENIVG 257

BjsipS    224  RADAVLGSWDLGMRGQPVWTCAATGFRLARFFTA--VH 259
               +A AV  SW    +             L  F     ++
Pflep:    258  KAFAVWMSWPE-PKLS----------HLPNFSRVGLIK 284
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Fig. 2: Sequence alignment of the signal peptidase of *P. fluorescens* (Pflep) and a symbiosis specific signal peptidase of *B. japonicum* 110*spc*4 (BjsipS). Full length deduced amino acid sequences are printed in bold, hydrophobic regions are underlined. The line in between represents the consensus sequence, conservative replacements are indicated by (+). The number of amino acids are indicated on both sides of each line. The fusion with '*phoA* in mutant 132 is in P-217. Amino acid residues known to be required for catalytic activity are marked by (*). Highly conserved regions A, B, C and D, are boxed.

Since the phenotypes of mutants 132 and 184 are clearly different from each other (Müller et al. 1995) it is concluded that the two genetic loci cannot functionally replace each other within one cell. Therefore we performed mixed coinoculation experiments on *Glycine max* to check whether strains 132 and 184 could cellularly complement each other. However, this was not the case. This result means that both genes must be involved in the processing of different (sets of) preproteins which are important for the symbiotic interaction with soybean.

Although at a lower rate, symbiosomes of mutant 184 induced soybean nodules can be isolated by saccharose (bouyant density) gradient ultracentrifugation. 2-D SDS-PAGE in combination with a Western analysis using a symbiosome protein specific antibody revealed that a number of proteins was detectable at a lower level, whereas other proteins were present in higher amounts (Fig. 1). The overall pattern of the protein spots resembled the results obtained after induced senescence.

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DIFFERENTIATION OF THE SYMBIOSOME COMPARTMENT IN PEA NODULE CELLS.

BREWIN, N.J., BOLANOS, L., DAHIYA, P. , GARDNER, C.D., HERNANDEZ, L.E., KARDAILSKY, I.V., RATHBUN, E.A., SHERRIER, D.J.
John Innes Centre, Norwich, NR4 7UH, Great Britain.

In this report, we describe the process of tissue and cell invasion by *Rhizobium* from the point of view of the increasing intimacy of cell surface interactions and we report some of our recent investigations relating to the development of the pea root nodule. Our main experimental approach has involved the use of antibodies as molecular probes for developmentally regulated cell surface components, coupled to the study of symbiotically-defective bacterial and plant mutants.

STRUCTURE AND DEVELOPMENT OF INFECTION THREADS

We have previously shown that the lumen of the infection thread can be considered as being an extension of the intercellular space (Rae et al. 1992). Rhizobia are separated from contact with the plant cell membrane by a sheath of primary cell wall that encloses the infection thread itself. The bacteria that grow and divide in the lumen of this tunnel are embedded in a matrix material apparently similar in composition to that found in intercellular spaces. Each bacterial cell is also cocooned in its own capsular sheath and genetic analysis indicates that the molecular composition of bacterial cell wall polysaccharides is very important for infection thread initiation and growth (Kannenberg, Brewin, 1994).

There seem to be two important questions relating to infection thread development. First, what are the physical forces that drive infection thread growth and, second, what are the molecular processes capable of controlling the colonisation of infection threads and intercellular spaces by potentially pathogenic "alien" bacteria? The first of these topics has been reviewed recently (Kannenberg, Brewin 1994) and in this presentation we will discuss only the second topic where our understanding is still rather sketchy. One emerging factor is the role of reactive oxygen species. We observe that the plant matrix glycoprotein recognised by monoclonal antibody MAC265 is capable of becoming insolubilised by cross-linking under oxidative conditions *in vitro*, confirming earlier observations made with *Phaseolus* cell suspension culture (Bradley et al 1992). We also

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observe by immunogold localisations that a lipoxygenase component is present in the infection thread matrix: this is interesting not only because it is the first reported extracellular localisation for a plant lipoxygenase but also because it is further evidence that reactive oxygen species may have an important function in controlling inappropriate infections. The recent finding that peroxidase is rapidly expressed in root hair cells in response to *Rhizobium* infection may also be relevant here (Cook et al 1995). The problem of tissue invasion by *Rhizobium* can thus be re-stated as the problem of not inducing a host defence response involving the release of active oxygen species. Is this achieved by "masking", i.e. the combined effects of capsular polysaccharide and lipopolysaccharide to create a protective sheath that cocoons the bacterial surface and prevents the exposure of cell surface signals that would otherwise trigger a host defence response? Alternatively, is it achieved by suppression, i.e. the release of signal molecules that could directly modify the normal host defence response?

A related problem concerning the control of infection thread growth may involve the physiological adaptation of both plant and bacterial cells which is presumably needed to accommodate the stresses associated with their mutual coexistence. Recently we isolated two classes of cDNA specifying thiolproteases from pea nodules (by using conserved oligonucleotides as primers in an RT-PCR reaction). We were surprised to find first that one of these genes had been previously identified as a drought-inducible gene in pea shoots (Guerrero et al 1990) and second that it was strongly expressed in the invasion zone of pea nodules, as revealed by *in situ* hybridization experiments. Perhaps these observations indicate that the intrusion of the infection thread into host cells, which might involve membrane stretch-activation, is physiologically similar to the loss of turgour associated with wilting and results in the expression of a similar set of plant genes.

ENDOCYTOSIS

Using free-living cultures of *Rhizobium*, we have recently conducted some experiments which may begin to throw light on the phenomenon of plant-microbial cell surface interactions and perhaps also on the essential role for boron in nodule development, which was first observed over seventy years ago (Brenchley,Thornton 1925). These experiments make use of the availability of monoclonal antibodies as molecular probes for individual bacterial and plant cell surface components. By mixing suspension cultures of (unencapsulated) rhizobia with nodule extracts containing plant matrix glycoprotein (a component of infection threads and infection droplets) we have demonstrated the attachment of matrix glycoprotein (MGP) to the bacterial surface (Fig. 1). Although stable to washing in 500mM NaCl, the attachment could be inhibited by 10mM sodium borate. We hypothesise that the presence of borate may function to inhibit binding of MGP to the bacterial cell surface and hence to promote a closer association between this surface and the plasma membrane of the infection droplet: such an interaction may be a precondition for endocytosis and the subsequent synchronised division of peribacteroid membrane with the division of intracytoplasmic bacteroids (Bradley et al 1986).

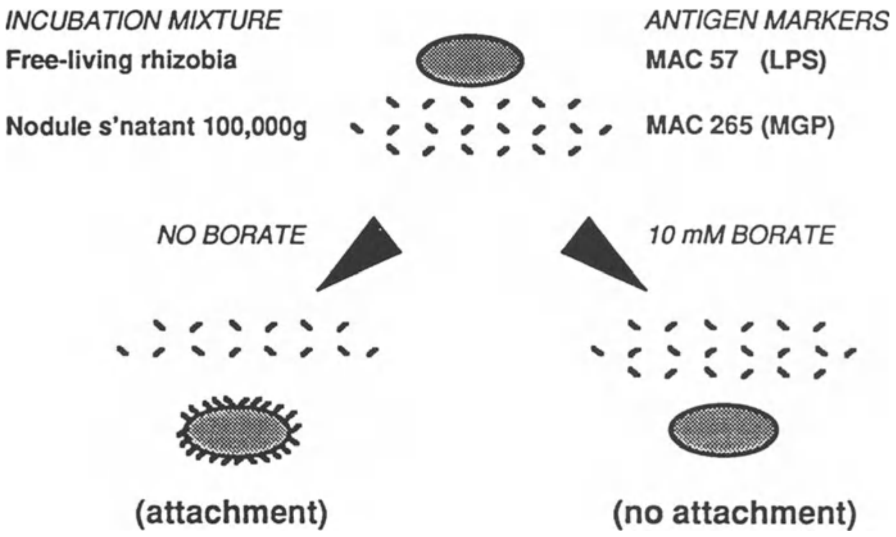

Figure 1. Effect of borate on the interaction between unencapsulated Rhizobium cells and a plant glycoprotein in the infection thread matrix.

DEVELOPMENT OF SYMBIOSOMES

Genetic and biochemical studies have clearly shown that the differentiation of bacteroids is controlled by host plant cell physiology, and by regulation of metabolic exchanges across the peribacteroid membrane (Verma et al 1994). As bacteroids differentiate, the composition of the peribacteroid membrane (PBM) gradually changes from that of plasma membrane to a hybrid of this with a specialised form of tonoplast membrane. In this laboratory, we have used monoclonal antibody probes for immunolocalisation studies of median longitudinal sections of pea nodules (Fig. 2) in order to monitor the progressive differentiation of the symbiosome compartment. On the plant side, the most useful epitopes have proved to be those identified by antibody MAC206 (recognising a plant glycolipid present on all plasma membranes but expressed strongly on all peribacteroid membranes, and by JIM18 which recognizes an inositol-containing lipid (probably a glycophosphosphingolipid) that is expressed on all plasma membranes and present on "juvenile" but not on "mature" forms of PBM (Perotto et al 1995). MAC266 and MAC254 recognise carbohydrate epitopes on a range of plant glycoproteins present in the Golgi apparatus, PBM and peribacteroid fluid: these glycoproteins are most abundant in mature symbiosomes and may reflect the induction of a new vesicle targetting pathway to symbiosomes. (One of these glycoproteins has recently been purified and shown to belong to a new class of nodule-specific lectin.) On the bacterial side, the three most useful antigens have proved to be epitopes of LPS of *R. leguminosarum* strain 3841. MAC57 recognises a constitutively expressed epitope, MAC203 an epitope expressed under low oxygen conditions and MAC 301 an epitope that is induced just prior to the induction of nitrogenase itself (Kannenberg et al 1994).

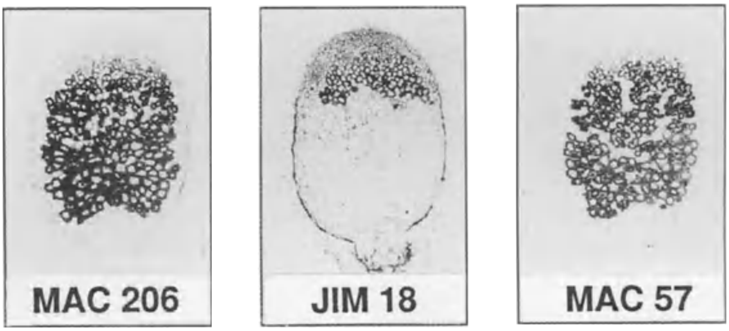

Figure 2. Silver-enhanced immunogold staining of serial longitudinal sections from a pea nodule (Perotto et al 1991). These sections reveal: the extent of Rhizobium infection (MAC57); the position of peribacteroid membranes (MAC206); and the transition point between juvenile and mature forms of PBM (associated with the loss of JIM18 epitope).

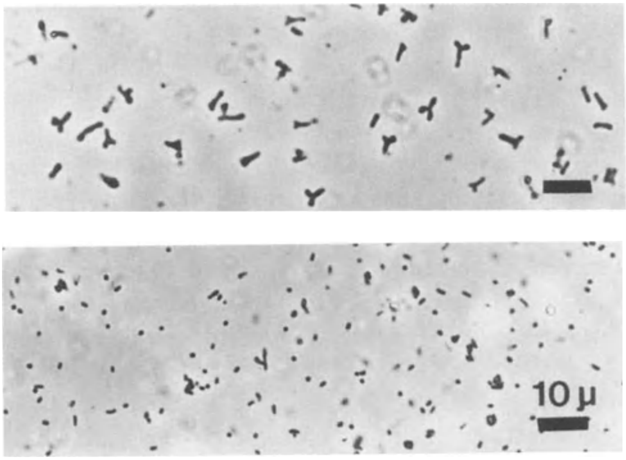

Figure 3. Phase-contrast micrographs showing bacteroids isolated from pea nodules of Sprint-2 and of Sprint-2 Fix$^-$*, a symbiotically defective mutant in which bacteroid development appears to be blocked at a juvenile stage (Borisov et al 1993).*

Using the antibody probes described above, we have investigated the differentiation of the symbiosome compartment for the pea line Sprint-2 Fix$^-$, a symbiotically defective mutant in which bacteroid development is apparently arrested at a juvenile stage (Fig. 3). This mutant is of particular interest because we observed by immunolocalisation studies that the JIM 18 epitope is permanently expressed on peribacteroid membranes throughout the nodule, implying that the transition from juvenile to mature forms of peribacteroid membrane does not take place. Intact symbiosomes were isolated from nodules of mutant and wild-type nodules in order to prepare PBM and bacteroid fractions and to test them by dot immunoassay with the antibody probes recognizing developmentally regulated plant and bacteroid epitopes. In addition, a 100,000g nodule membrane fraction (consisting largely of microsomal and plasma membranes) was prepared from the nodule homogenate after removal of symbiosomes by low speed centrifugation. The results of dot assays (Fig. 4) support the hypothesis that symbiosome development in Sprint-2 Fix$^-$ nodules is blocked at an early stage. This was true both for plant developmental markers (i.e. there was no reduction of JIM18 antigen but reduced expression of MAC266 and MAC254 antigens on PBM), and for bacterial antigens (lack of expression of MAC301 LPS epitope and nitrogenase antigen). Interestingly however, the bacteroids from Sprint-2 Fix$^-$ nodules did express the MAC203 LPS epitope which is expressed in a low-oxygen microenvironment, implying that this aspect of nodule development is relatively unimpaired in the pea mutant. The low level of release of LPS antigens into the peribacteroid membrane fraction of Sprint-2 Fix$^-$ was further evidence of the undifferentiated state of these bacteroids. It was also noticeable that the MAC266 and MAC254 antigens were strongly represented in the microsomal pellet fraction for Sprint-2 Fix$^-$, suggesting that these antigens were still being expressed although not targetted to the symbiosome compartment.

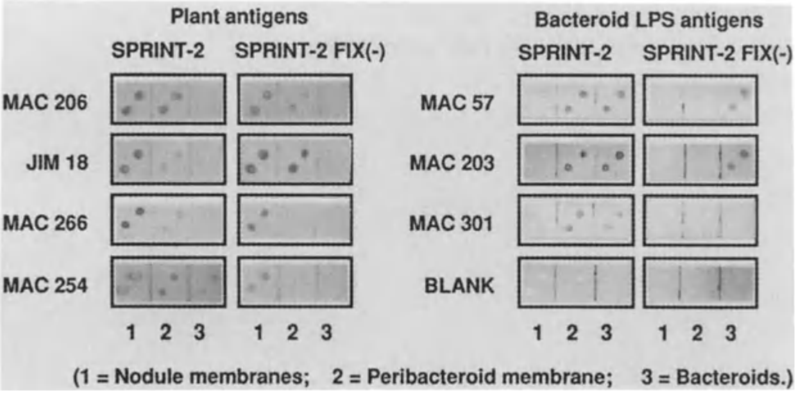

Figure 4. Immunoassay for symbiosome antigens from Sprint-2 and Sprint-2 Fix peas.

CONCLUSIONS

From these studies, we conclude that the first phase of tissue colonisation by *Rhizobium* (i.e. the development of the infection thread) involves reorganised plant cell wall growth and the second phase (the development of the symbiosome) involves reorganised membrane biogenesis. It would appear that membrane differentiation and vesicle targetting for infected host cells may be the key to metabolic specialisation for the endosymbiotic bacteroids themselves. Our long-term goal is therefore to investigate how membrane trafficking pathways are reprogrammed to create a new compartment (the symbiosome) performing a new metabolic function (biological nitrogen fixation).

ACKNOWLEDGEMENTS

We thank Silvia Perotto and Elmar Kannenberg for their previous contributions to the research described here, and we are grateful to Igor Tikhonovich for supplying samples of Sprint-2 and Sprint-2 Fix$^-$ peas. We acknowledge financial support from the UK-BBSRC, the John Innes Foundation and Commonwealth Scholarship Commission.

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PLANT GENETICAL CONTROL AT THE CRUCIAL STEPS OF SYMBIOSIS

TIKHONOVICH, I.A. (1), BORISOV, A.Y. (1), CHVABAUSKENE, I.A. (1), KAMARDIN, N.N. (1), KRAVCHENKO, L.V. (1), LEBSKY, V.K. (1), MINCHIN, F. (2), MORZHINA, E.V. (1), ROMANOV, V.I. (3), SKOT, L. (2), TCHETKOVA, S.A. (1), TSYGANOV, V.E. (1).
(1) Res. Inst. for Agricultural Microbiology, St. Petersburg, Russia.
(2) Inst. of Grassland and Environmental Research, Plas Gogerddan, Aberystwith, Dyfed SY 23 3 EB, UK.
(3) Bach Inst. Biochemistry, Moscow, Leninsky pr. 33, 117071, Russia

Study of plant genetic control of symbiosis structures is becoming more and more urgent for understanding plant-microbe interaction. Nowadays a lot of nodulins and other proteins involved in plant-microbe interaction have been revealed, and the structure of corresponding genes has been described. The lack of knowledge about the structural basis for the symbiotic plant genes is very clear now when researchers try to understand not only the functions of single genes and their products, but to develop the theory of the creation of the symbiosome during symbiosis. Symbiosome formation is seen as a crucial event in symbiosis so plant mutants impaired in this process are of special interest. Many sym mutations are most likely acting as regulatory elements integrating the process of symbiotic organelle formation.

In our laboratory, together with colleagues from Russia and abroad, we have created genetic models for studying symbiosome formation in pea. Initially, the purpose of our work was to obtain symbiotic pea mutants and characterize them in order to determine the elementary stages of symbiogenesis. We received a lot of Nod$^-$ mutants but Fix$^-$ ones, used as a model for the symbiosome development, were rather rare. To obtain the symbiotic mutants by EMS mutagenesis, two pea genotypes were used - Sprint-2 and SGE lines (Berdnikov et al., 1989). Starvation of mutated plants in nitrogen free medium was the main characteristic for screening M_2 population. All Fix$^-$ mutants lacked acetylene reduction activity and their physiological characteristics demonstrated the ineffective symbiosis. Morphologically, nodules of these mutants looked very different. Sprint-2Fix$^-$ (symX) and SGEFix$^-$-1 have numerous white nodules; SGEFix$^-$-2 forms different morphological types of nodules simultaneously present on one root (see abstract Tsyganov et al., "Isolation of..."). Mutations in SGE are connected with the development and functioning of the infection threads and their relationships with symX and sym13 are to be elucidated. In our collection, we also have Fix$^-$ mutants received from other laboratories. In our work we used the well known early senescent E135fFix$^-$ (sym13) mutant from Dr. LaRue (Kneen et al., 1990) and FN1Fix$^-$ from Dr. Jacobsen (Postma et al., 1990). According to allelic test, all isolated Fix$^-$ mutants were non-allelic and Fix$^-$ mutation in Sprint-2 was localized on the 3d pea chromosome.

Electron microscopic study showed that all Fix$^-$ mutants have clearly visible deviations from the normal symbiogenesis process. In the effective variants, well differentiated

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Fig. 1. Sprint-2 + 1026

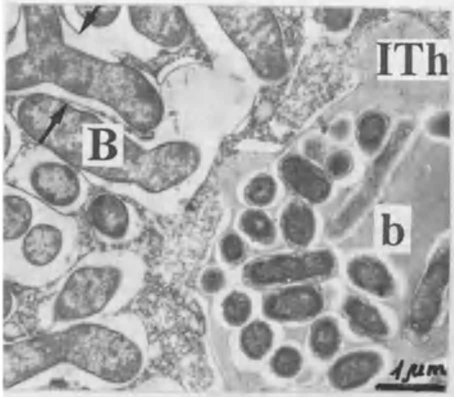


Fig. 2. Sprint-2Fix$^-$ + 1026

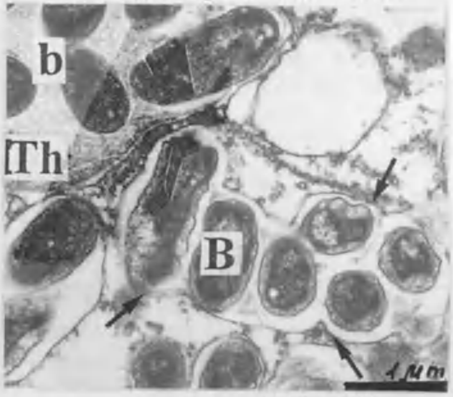


Fig. 3. E135fFix$^-$ + 1026

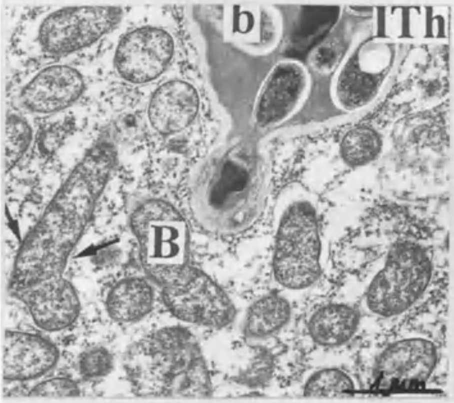


Fig. 4. RBTFix$^-$ + 1026

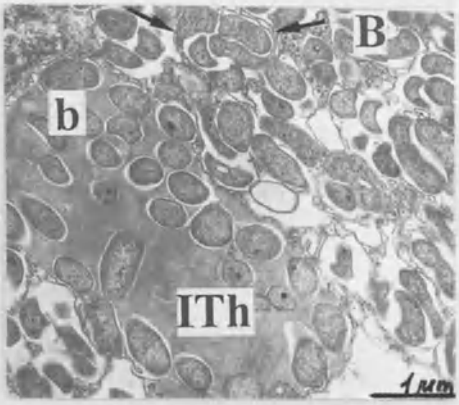


Fig. 5. Sprint-2Fix$^-$ + 1064Fix$^-$

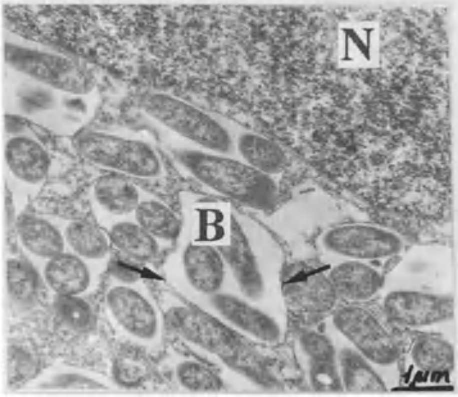
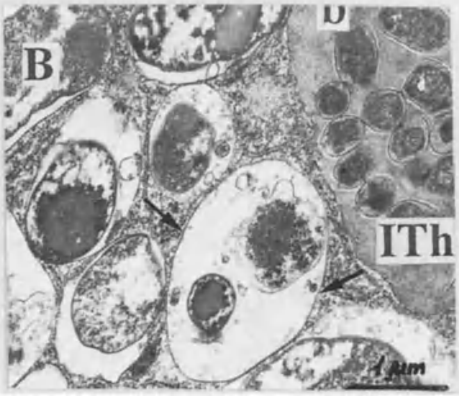
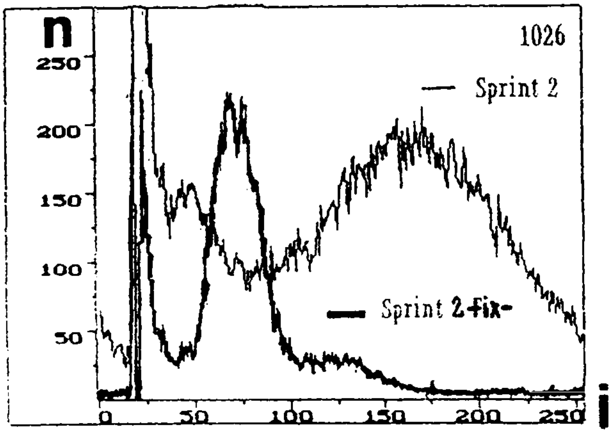


Fig. 6. E135fFix$^-$ + 1064Fix$^-$



pleiomorphic Y-shaped bacteroids are formed that clearly differ from bacteria in infection threads (Fig.1). The type of symbiosomes in the nodules of Sprint-2Fix$^-$ is absolutely untypical for pea (Fig.2): bacteroids in the symbiosomes and bacteria in the infection thread hardly differ from one another. Due to the continuous division, or to the aggregation of newly formed symbiosomes, not one but several bacterial cells are packed within one PBM. At the same time both in mutant FN1 and in E135f (Fig.3) bacteroids undergo early stages of differentiation coupled with the early senescence; probably due to the enhanced plant pathogenic response. The most striking phenomenon of the Sprint-2Fix$^-$ is the absence of bacteria transformation into bacteroid form. Study of several characteristics of this mutant showed that its nodules, after inoculation with the effective strains, have no leghaemoglobin; there is no normal nitrogenase enzyme. This impairment in bacteroid transformation is not connected with the oxygen concentration in the nodule because the oxygen barrier is present in this mutant. Isolated bacteroids of the parent cultivar are much larger and have the branching form, while in the mutant, the bacteroid-like cells are much smaller and, in form, are closer to the free living bacteria. Sprint-2Fix$^-$ shows that the host-plant controls the process of bacteria multiplication. To study this in more detail, we used the microspectrophotometric measurement of DNA content in bacteroid and free-living bacteria. The cytofluorymeter with the argon laser allows the measurement DNA content in the single bacteria cell. Comparison of the histograms received for the Sprint-2Fix$^-$ bacteroids and for the free-living cells showed no differences, while in the parent most of the cells have several times more DNA in bacteroids than in the free-living bacteria. Increasing the DNA content is probably due to the endoreplication process which takes place during the bacteroid formation (Fig.7). Single plant gene controls the process of bacteria endoreplication. In the absence of this action the microorganisms released from the infection thread continue to divide and the multicell symbiosomes are formed.



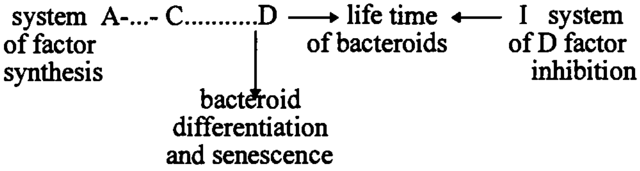
n - number of cells measured

i - amount of DNA per one cell

Fig.7. DNA content in the bacteroids from Sprint-2 and Sprint-2Fix- nodules, inoculation by effective strain 1026.

The existence of several non-allelic Fix$^-$ mutants raises the question about the hierarchy of their action during the symbiogenesis. Comparative morphological and physiological study of Sprint-2Fix$^-$ (symX), E135f (sym13) and RBT line combining both mutations (symX and sym13) showed that symX is expressed prior to sym13 (see abstract: Borisov et al., "Identification of..."). The most simple explanation is that Sprint-2Fix$^-$ is mutated in the gene which controls the creation (synthesis) of "differentiation factor". Other plant-bacterial mutant combinations (E135f+1064Fix$^-$; Sprint-2Fix$^-$+1064Fix$^-$; Sparkle+1064Fix$^-$; E135f+1026Fix$^+$), both of plant and bacterial origin, are characterized by the early senescence. In all cases, formation of bacteroids morphologically different from bacteria (larger in size, unformed, with lighter electron density matrix) and unable to fix nitrogen, are characteristic for all Fix$^-$ variants irrespective of plant or bacterial reason of ineffectivity. The recessive mutation in E135f manifests itself as an intensive and rapid differentiation resulting in early senescence. Its unlikely for the recessive mutation to increase the function, thus it seems more logical to propose differentiation as a two component gene system (Fig.8). Comparative study of the Fix$^-$ pea mutants points out the action of these two genes is controversial. If the first gene D forms or accumulates the substances (or even compartments) determining the differentiation at the normal concentration or senescence when abundant, I gene in the dominant state decreases the initial level of D factor to the normal level, or/and controls the distribution of D factor through the nodule tissue. The possibility to sustain bacteroids in a nitrogen-fixing state depends on the balanced action of these two genes - D and I. Most probably the plant genes responsible for differentiation/senescence carry out such control by interacting with a bacterial factor, not necessarily a symbiotic one. Stressing this last point is the fact that most important symbiotic genes - nifA, and fixC, are expressed in the bacteroids of Sprint-2Fix$^-$, as it has been shown using nifA::lacZ and fixC::lacZ reporter fusion.

Fig. 8. Plant control of bacteroid differentiation and senescence


system of factor synthesis
A-...- C...........D
life time of bacteroids
I system of D factor inhibition
bacteroid differentiation and senescence

Genes and mutations	Differentiation factor	Bacteroid phenotype
Fix$^-$ Sprint A ... C — x — D ⟶	is absent	no differentiation, long life in cytoplasm
Fix$^-$ Sparkle ⟵ x — I	is accumulated in large amounts because of the damage in the inhibition system	early senescence, differentiation is clearly visible
Fix$^-$ / Fix$^-$ RBT A ... C — x — D ⟶ ⟵ x — I	is absent	low level differentiation

All these speculations raise the question about the nature of D-factor. Assuming the scheme of D and I-factor interaction (Fig.8) genotype of Sprint-2Fix$^-$ may be understood as a mutation resulting in the lack of mature "differentiation factor" and therefore the low content of D-product along with thefunctional I. This would explain the absence of differentiation and normal nodule senescence. In the E135f most probably there is a lack of I-factor so that D provides for the accumulation of "differentiation factor", resulting in a very rapid differentiation of bacteroids and leading to rapid senescence. In the combination of these two recessive mutations in one genotype RBT (Fix$^-$ /Fix$^-$), where no differentiated bacteroids are found, (Fig.4) indicates that in spite of the absence of the inhibition system, there is no active "differentiation factor".

The same scheme allows us to explain bacterial ineffectivity in 1064Fix$^-$ strain that seems to have an increased sensitivity of bacteroids to the "differentiation factor" (Fig.5,6). Most dramatically "differentiation factor" manifests itself in the variant E135+1064Fix$^-$, and in this case, the accumulation of "differentiation factor" and super sensitivity of the strain, leads to the initiation of the differentiation with immediate degradation of the bacteroids.

Analogously to early stages of symbiosis transformation into bacteroids may also be connected with some signal compounds - the flavonoid-like substances being the most probable candidates in this respect. The other possibility is the dependence of transformation on the changes in metabolic and nutritional status. The HPLC profiles of the nodulated roots following methanol extraction in parental cultivars - Sprint-2 and Sparkle and ineffective mutants Sprint-2Fix$^-$, Sparkle E135f and RBT at the age of four weeks differed considerably. The HPLC pattern of the unrelated parents was markedly different from that of all three mutants. The general type of the HPLC profile seems to reflect the symbiotic status on the whole, effective or ineffective, while the quantity of some peaks (26-27 min.) correlated with the individual mutation and with the symbiosome development. The level of "peak 26 min" is maximal in Sprint-2Fix$^-$, minimal in E135f, and intermediate in the double mutant RBT, being a marker of a particular mutation symX. The other possible reason, and also the mechanism of host control over symbiosome, lies in the changing of carbon nutrition when bacteria find themselves dependent upon the photosyntate supply. Differences between mutants and parents were revealed in respect to the substances that are not of signal nature but play an important role in carbon and nitrogen metabolism in the nodules. Again the amount of plant supplied carbon substances - glucose and sucrose - reflected the symbiotic status of the nodules but not the individual mutation which was much higher in all three mutants (Table 1).

Table 1. Content of soluble carbohydrates in ethanol extracts from equal amounts of whole, normal and ineffective nodules of pea.

	umol/g/fr.weight		
Plant line	Ononitol	Glucose	Sucrose
Sparkle	5.87	1.55	2.71
E135f	4.68	3.49	7.69
Sprint-2	4.30	1.62	4.22
Sprint-2Fix$^-$	0.45	2.42	6.88
RBT	0.81	1.46	2.26

The only substance whose pattern could fit into the proposed scheme was a sugar-like compound, ononitol, typical for pea nodules. Ononitol, abundant in both parental cultivars Sprint-2 and Sparkle and also in Sparkle's mutant E135f, was 10 times less in Sprint-2Fix$^-$. Taking into consideration that Sprint-2Fix$^-$ differs from E135f in non-differentiation of bacteroids, low ononitol content seems to be a marker of a particular mutant allele symX and not of the Fix$^-$ status of nodules. The amount of ononitol in the double mutant RBT, being closer to the Sprint-2 than to the Sparkle mutants, strengthen this idea besides pointing out the fact that symX expresses earlier in symbiosis than sym13. Dr. Skot proposed earlier that ononitol may play an important but unknown role in nitrogen fixation. Our data shows that ononitol is also involved in the transformation of bacteria into bacteroids.

Conclusion

The collection of symbiotic pea mutants allowed us to demonstrate at least two elementary steps in the process of symbiosome formation and to attribute these steps to the particular gene alleles - symX and sym13. We propose a hypothetical model for understanding of the role of these genes in symbiosome formation and point out elementary biochemical traits that characterize the manifestation of these genes.

Markers of an individual Fix- mutation :

1. the HPLC peak at 26 minute in the flavonoid fraction of nodulated roots;
2. ononitol content in the nodules.

Markers of the general inefficient symbiotic status:

1. general profile of HPLC peaks of the flavonoid fraction of nodulated roots;
2. content of sugars (sucrose and glucose) in the nodules.

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Acknowledgments

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Legend:

B - bacteroid; b - bacteria; ITh - infection thread.

BIOGENESIS OF THE PERIBACTEROID MEMBRANE IN ROOT NODULES: ROLES OF DYNAMIN AND PHOSPHATIDYL-INOSITOL 3-KINASE

VERMA D.P.S., GU X. AND HONG Z.
Department of Molecular Genetics and Plant Biotechnology Center,
Ohio State University, Columbus OH 43210

Rhizobia in the infected cells of root nodules are compartmentalized inside a membrane, peribacteroid membrane (PBM), where they are differentiated into bacteroids and begin fixing nitrogen. Since bacteria are never free inside the plant cell during symbiosis, the PBM serves as an interface between the two organisms and its integrity is vital for symbiotic nitrogen fixation. As bacteria are released from the infection thread they are endocytosed by the plasma membrane of the host and this membrane proliferates to enclose bacteria individually or in a group to eight per envelope. This necessitates a tight coordination of PBM biogenesis with the bacterial cell division and the number of bacteroids per envelope reflects when cell division ceases following endocytosis. This event is determined by the host. Although derived from the plasma membrane (PM), the PBM undergoes significant chandes and becomes a mosaic membrane having properties in common with the vacuolar and the PM (Verma, 1992). Thus, vesicular traffic in root nodules must be unique to direct appropriate PBM nodulins and the peribacteroid fluid proteins to their proper sites. The plant genetics data (Tikhonovich et al., 1995) is consistent with this interpretation. The mechanism controlling PBM biogenesis and proliferation is poorly understood. Recent studies on plant and bacterial genetics have shed some lights on biogenesis of this compartment and suggest that both bacteria and the host plant control the synthesis of PBM and its effectiveness in symbiosis.

The PBM and peribacteroid fluid (PBF) proteins are synthesized on rough ER and pass through Goldi following vasicular traffic. We have isolated a number of small GTP-binding protein genes from Vigna and soybean and demonstrated that these genes play an important role in PBM biogenesis (Cheon et al., 1993). Expression of these genes is enhanced during early nodule development suggesting their role in vesicular traffic. Because each bacteroid or a group of them is enclosed in PBM the membrane biogenesis is significantly increased in the infected cells of root nodules (Verma et al., 1978). We created conditional dominant mutations in these genes by antisense expression under the control of leghaemoglobin promoter. Transgenic hairy roots containing the desired antisense construct were nodulated by *Bradyrhizobium japonicum* and the main roots were removed to support the plant on transgenic roots. This chimeric system saves significant time in transgenic work which otherwise takes a significant time in legumes.

Antisense rab1 experiments demonstrated an arrest in membrane flow from the ER to Golgi leading to break up of PBM and the release of bacteroid in the cytoplasm or vacuole. The central vacuole persisted in these cells which is normally absent in

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soybean. The arrest in membrane flow also retarded the enlargement of the infected cells as well as the infected cells and nodules remained very small in size. A very low level of nitrogen fixation activity was,detected in these nodules. On the other hand, the antisense expression of rab7 resulted in normal size nodules, but the nature of the PBM compartment seems to be altered. This protein is known to affect post Golgi traffic and, consequently, a retardation in the flow of proper membrane proteins and some PBF proteins makes this compartment a lytic compartment where bacteroids are often degenerated. This event occurs in the periphery of the nucleus where a numder of multivesicular bodies were observed (Cheon et al., 1993). This study suggested that PBM biogenesis follows a vesicular traffic route from ER to Golgi and is consistent with the synthesis of nodulin-24 and nodulin-26, two major PBM nodulins, studied in our laboratory. The PBF proteins may follow either the path of extracellular proteins or those of vacuolar proteins. A number of vacuolar proteins are known to be accumulated in this space. In addition, PBF also contains proteins secreted by bacteria and the presence of these proteins may be important for the proper PBM functioning (see below).

The phospholipid composition of PBM may be unique although its density is similar to that of PM (Verma et al., 1978). We have demonstrated the presence of phosphatidylinositol 3-kinase and a nodule-specific form of this enzyme was identified (Hong, Verma, 1994). The PI 3-kinase specifically phosphorylates the D-3 position of the inositol ring to generate phosphatidylinositol 3-phosphate (PI3-P). The latter is involved in signal transduction and vesicular transport of proteins to vacuoles. Three genes encoding PI3-kinase type proteins have been identified in yeast and the VPS34 gene product has been demonstrated to be involved specifically in vacuolar protein sorting. We recently isolated two homologs of VPS34p from soybean and demonstrated that the expression of one of them is increased during nodule development while that of the other one decreases during the period when extensive PBM biogenesis occurs. This reciprocal inducibility of PI3-kinase genes with a corresponding increase in activity of this enzyme suggests that the nodule-form of the PI3-kinase enzyme may participate in PBM biogenesis (Figure 1). This enzyme is associated with the membrane fraction in root nodules but is not a part of the PBM. Soybean PI3-kinase expressed in *E.coli* exhibited catalytic activity and the protein does not have any trans-membrane domain suggecting that this enzyme becomes associated with the membrane with the help of some other protein or is modified post-translationally. A decrease in activity of this enzyme in mature nodules coincides with the lack of membrane turn over and the lack of Golgi activity at this stage of nodules (Verma et al., 1978).

In animals, a GTP-biding protein, dynamin has been implicated in endocytosis. We searched for a homolog of this protein with the possibility that this mechanism may be conserved in eukaryotes. We isolated two distinct genes from soybean encoding dynamin-like proteins (Gu and Verma, unpublished data). The soybean dynamin lacked the carboxy end of the animal dynamins but had significant homology at the amino terminus including the GTP-binding domain. Northern blot data suggested that the expression of this gene is enhanced during early nodule development with highest expression being in root tips. We expressed soybean dynamin cDNA in E.coli and raised antibodies against the purified protein. Immunocytological localization of this protein did not reveal any specific locus in root nodules, however, this protein was found to be specifically associated with vesicles destined for cell plate formation.

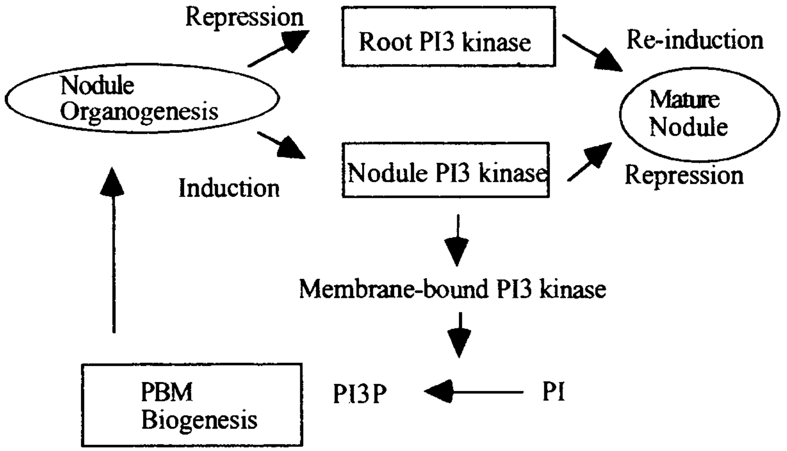

Figure 1: Induction and role of nodule-specific PI3 kinase in PBM biogenesis

This data suggests that dynamin may not be involved in endocytosis in plants, instead this protein may have been recruited for exocytosis of vesicle contents for deposition on the cell plate. A low level of this protein is present in nodules and elongation zone of root as shown by western blot data.

Plant genetic studies from Tikhonovich's group (Tikhonovich et al., 1995; see this volume) have identified a number of pea mutants that are defective in PBM biogenesis suggesting that some specific nodulins are involved in the biogenesis of the PBM compartment in root nodules. These mutants do not affect biogenesis of any other membrane and their gene products may specifically interact with some components from bacteroids. A number of mutants have been identified in ***Bradyrhizobium*** in several laboratories that either fail to endocytose from the infection thread or form defective PBM compartment. Two of these have been shown to encode membrane proteins as identified by the studies on Tn-*pho*A insertions in bacteria (Muller et al., 1995). These proteins are specific signal proteases processing proteins destined for secretion. Three such proteins were identified by Werner's group using antisera prepared in our laboratory. This suggests that specific proteins are secreted in the PBF that play a role in biogenesis and/or the integrity of the PBM compartment. These studies also demonstrated a gene-for-gene interaction in the biogenesis of functional PBM and any perturbation in this interaction leads to an abnormal membrane which is not fully effective in symbiotic nitrogen fixation. These studies further confirm the importance of PBM in symbiosis of plants with rhizobia and suggest that biogenesis of this membrane must be taken into consideration in any attempts to force endo-symbiosis of nitrogen-fixing organisms with non-legumes, such as monocots. This membrane interface between a procaryote and the host plants allows the two organisms to be temporally together without eliciting a host defense reaction which would otherwise kill the bacteria or lead to the death of the plant cell.

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EXPRESSION OF LEGUME LECTIN GENES

DE PATER, B.S.(1), BAUCHROWITZ, M.A.(2), DÍAZ, C.L.(1),
BARKER, D.G.(2), TRUCHET, G.(2) AND KIJNE, J.W.(1)
(1) Inst. Molecular Plant Sciences, Ctr.Phytotechnology RUL-TNO,
Leiden Univ., Wassenaarseweg 64, 2333 AL Leiden, The Netherlands.
(2)Laboratoire de Biologie Moléculaire des Relations Plantes-Micro-organismes, INRA-CNRS, BP 27, 31326 Castanet-Tolosan, France.

Legume lectins

Legume lectins are a homologous group of sugar binding proteins. Sugar binding is specific, since, for example, pea lectin (PSL) binds mannose and soybean lectin (SBL) binds N-acetyl-galactosamine. Legume lectins are either dimeric or tetrameric, with each monomer possessing a single sugar binding site. As a result, these proteins are able to crosslink sugar-coated cells or glycoconjugates or can themselves be crosslinked by biantennary saccharides (Dessen et al. 1995).

Usually, legume lectins are abundant in seeds, where they are stored in protein storage vacuoles during seed development. Considerably smaller amounts of lectin are present in legume roots, where lectins can also be secreted. In legume roots, lectins and antigenically related proteins can be found in vacuoles (Vodkin, Raikhel, 1986), in cell walls (Díaz et al. 1984) and at the tip of growing root hairs (Dazzo et al. 1978, Stacey et al. 1980, Law, Strijdom 1984, Díaz et al. 1986). Secreted lectin is able to bind to surface polysaccharides of *(Brady)rhizobium* bacteria, and contributes to the formation of rhizobial aggregates at the tip of host plant root hairs (Dazzo et al. 1984, Kijne et al. 1988). Furthermore, from experiments described by Halverson and Stacey (1985), Díaz et al. (1989) and Brelles Marino et al. (1993) it can be concluded that root lectins stimulate rhizobial infection of the host plant root by an as yet unknown mechanism. The sugar-binding activity of lectin seems to be essential for this function (Van Eijsden et al. 1995).

Lectin genes

Legume lectins are encoded either by a single gene (pea) or by a small gene family (for example, alfalfa, bean, soybean, *Dolichos*). The gene products are lectin precursors, which are subsequently processed on their way to the vacuole or to the cell surface. Legumes may also contain lectin-like genes. Some of these encode plant defence proteins such as arcelin and α-amylase inhibitor (Mirkov et al. 1994). These proteins are unlikely to represent sugar-binding proteins, since they lack one or more amino acid residues known to be essential for sugar binding. The properties of proteins encoded by *blec* (the "pea bud lectin", Mandaci, Dobres 1993) and *nlec1* (the "pea nodule lectin", Kardailsky, Brewin 1994) have yet to be determined. Other lectin-like genes are pseudo-genes due to the presence of extensive deletions, frame shifts and the presence of stop codons (for example, Bauchrowitz et al. 1992, Kaminski et al. 1987).

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We have studied the expression of lectin genes using two different systems, transgenic tobacco plants and transgenic *Medicago varia* plants. With the first system, seed-specific and developmentally regulated expression of the pea lectin gene (*psl*) has been analyzed. With the second system, information has been obtained about the expression of *Medicago* lectin genes (*Mtlec1, 2* and *3*) in roots during the establishment of the symbiotic association with *Rhizobium*.

Seed-specific gene expression

The abundance of pea lectin (PSL) in transgenic tobacco seeds is due to high expression of the *psl* gene (Higgins et al. 1983). Shortening of the *psl* promoter to the 115 bp of the 5' flanking sequence significantly lowered the level of expression, but seed-specificity and developmental control were unaltered (De Pater et al., manuscript in preparation). Similar results were found for a soybean lectin gene (*Le1*), when tested in transgenic tobacco (Lindstrom et al. 1990). The 115 bp *psl* promoter fragment contains a 22 bp-region with three overlapping TGAC-like motifs. Such motifs have been shown to be binding sites for basic/leucine zipper transcription factors (bZIP proteins)(for example, Katagiri et al. 1989). A trimer of this 22-bp region, when coupled to a heterologous minimal promoter, conferred a high level of reporter gene expression in seeds of transgenic tobacco, and only a low level of reporter gene expression in root, stem and leaf. Seed expression increased during seed development and was observed in the endosperm as well as in the embryo. However, a few days after germination, reporter gene expression appeared to be dramatically lowered. This expression pattern is similar to that conferred by a 2000-bp fragment of the *psl* promoter (De Pater et al. 1993).

The 22-bp region contains a binding site for the cloned bZIP proteins TGA-1a and Opaque-2 (O_2)(De Pater et al. 1994). Both proteins bound to the odd base palindromic C-box sequence ATGAGTCAT, present in the 22-bp region. Specific mutations in this box resulted in strongly decreased binding of the bZIP proteins, and eliminated seed-specific gene expression. Measurement of binding affinities showed that O_2 strongly bound to the odd base C-box, in contrast to TGA-1a. O_2 is a maize bZIP regulatory protein that plays an essential role in controlling the expression of seed storage protein genes (Motto et al. 1989). Based on our results, we expect the pea protein involved in high seed expression of *psl* to be a bZIP protein with high binding affinity for the odd base C-box, as is the case for O_2. TGA-1a is a tobacco transcription factor that binds to the A-domain of the Cauliflower Mosaic Virus 35S-promoter which confers expression mainly in root (Benfey et al. 1989). Low expression of *psl* in vegetative tissues could be conferred by a factor with low binding affinity, such as TGA-1a.

Gene expression and nodulation

Medicago truncatula contains three lectin genes, *Mtlec1* and *2* (Bauchrowitz et al. 1992) and *Mtlec3* (Bauchrowitz et al. 1995). *Mtlec2* is probably non-functional since a frameshift (insertion of two nucleotides) leads to premature translation termination. The developmental pattern of lectin gene expression throughout various stages of the *Rhizobium*-legume interaction was studied by use of promoter-*gusA* fusions for each of the three lectin genes. In non-inoculated roots, *Mtlec1* and *Mtlec3* are expressed at a low level in inner cortical and vascular root tissues, respectively. Following inoculation with compatible rhizobia, both promoters are active in dividing cells of root nodule primordia, whether elicited by rhizobia or by purified lipochitin oligosaccharides (Nod factors). Each of the three lectin promoters is active in mature nodules, in which gene expression was restricted to non-infected cells of both central and peripheral tissues (Bauchrowitz et al., submitted). In addition to a role of lectins in direct interactions with rhizobia, these observations suggest that lectins could be implicated in plant developmental processes.

Concluding remarks

The research strategies mentioned above are clearly complementary. In both cases, many questions remain to be answered. Several future (and ongoing) experiments aimed at examining these questions are (i) testing of *psl* promoter constructs lacking the 22-bp region for activity in seed and root, (ii) identification of putative bZIP transcription factors involved in (seed-specific) *psl* expression, (iii) isolation and characterization of *Medicago* lectins, (iv) testing for the presence of *Medicago* lectins in nodule primordia and nodules, (v) testing of *psl* expression in transgenic alfalfa, and, last but not least, (vi) testing of a root-lectin deficient pea or alfalfa mutant for nodulation capacity.

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GENETIC EXPERIMENTS WITH PEA MUTANTS PENDING SYMBIOSIS STUDIES

SIDOROVA K.K., SHUMNY V.K., UZHINTSEVA L.P.
Institute of Cytology and Genetics, the Siberian Department of the
Russian Academy of Sciences, Novosibirsk, Russia

Nitrogen fixation by legume plants is a result of an overall symbiotic interaction with the nodule bacteria *Rhizobium*. Mutants of both organisms may be useful for identification of the genes controlling this process. About 30 genes controlling stages of development and functioning of nodules in pea have been determined by genetic analysis of symbiotic mutations (LaRue, Weeden, 1992).

We report the results of a genetic analysis and grafting of three symbiotic pea mutants. A supernodulating nts mutant, K301, and another, K287 with ineffective nodules, were induced by NEU from the cultivar Ramonsky 77; the non-nodulating mutant K1005m by EMS from mutant K511 which had earlier been induced in the cultivar Torsdag.

The plants were grown in the greenhouse on a ceramsite : vermiculite mixture as a substrate. The supernodulating nts mutant K301 was grown with nitrogen added to the standard mineral mixture as nitrate salts. The mutant with ineffective nodules K287 and the non-nodulating mutant K1005m were grown away from nitrogen. The starting dose of nitrogen was 0.2% of the standard. The other components of the mixture were as recommended by standard techniques. The greenhouse temperatures was 20-22^{0}C during the day and 14-15^{0}C in the night. The light/dark ratio in hours was 16/8. The seedlings were inoculated by *Rhizobium leguminosarum* strain 250a. Nitrogenase activity was determined by the acetylene reduction method.

Graftings were performed as described by Postma et al.(1988).

In the supernodulating mutant K301, the number of nodules per plant was about 1000, no matter whether nitrates were present in the substrate or not (Fig.1). The nitrogenase activity of the mutant was 15-20 and more times higher than that of the original cultivar when both were grown with nitrates and just 3-5 times when no nitrates were added.

The supernodulating mutant K301 has a fasciated stem, the mutant with ineffective nodules K287 has shortened internodes. In both mutants, the stem form character is inherited together with the symbiotic character due to the pleiotropic effect of the mutant gene.

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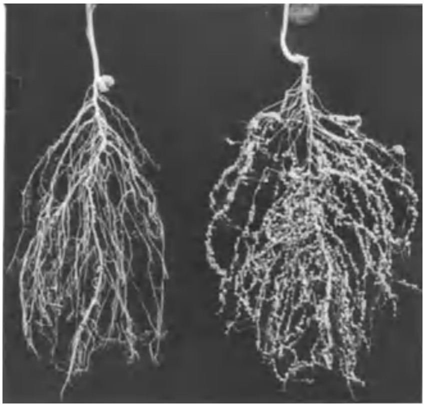

Fig. 1. Left: the root system of the initial cultivar Ramonsky 77;
Right: the root system of the supernodulating nts mutant K301.

The three mutants result from recessive monogenic mutations (Table 1).

Table 1. Segregation in F_2 populations from crossing the mutants to the original cultivars

Hybrid	Segregation ratio "original : mutant" (3:1)	χ^2	P
K301 x Ramonsky 77	106 : 41	0.655	0.50 - 0.25
K287 x Ramonsky 77	133 : 53	1.211	0.50 - 0.25
K1005m x K511	44 : 14	0.01	0.95 - 0.90

In the analysing crosses, the segregation ratio was 1:1 in the three hybrids.

Results of enter se crosses of the mutants confirmed that these mutations were due to different genes. The K301 x K287 cross gave in F_2 a dihybrid segregation ratio of 9:3:3:1 (χ^2 = = 2.358, P = 0.75-0.50). The cross K1005m x K301, and K1005m x K287 no otherwise, gave a ratio of 9:3:4 (χ^2 = 2.539, P = 0.50-0.25; χ^2 = 5.288, P = 0.10-0.05).

The reciprocal graftings between the mutant and original cultivars on the stems and roots showed that in K301 supernodulation was controlled by factors originating in the stem, whereas in K1005m and K287 the symbiotic characters were controlled from the root.

We determined chromosomal localisation of the gene controlling supernodulating in K301 (here denoted as *nod*$_4$) by using the tester lines WL1238, WL1132 and WL741 kindly provided by Dr Blikst from his genetic collection.

Results of the investigation showed that the gene *nod*$_4$ is located at chromosome 5 (Tables 2,3). At the "K301 x WL1238" cross in the F_2 population, a monohybrid segregation was noted for the mutant, namely *nod*$_4$, and marker, namely *gp* and *cp* genes.

Table 2. Analysis of joint segregation in F_2 for *nod*$_4$ and the marker genes *gp* and *cp* in crossing the mutant with the tester line WL1238

Phenotype in F_2	Number of plants	χ^2	Recombination fraction
	nod$_4$ - *gp*		
Nod$_4$ - *Gp-*	173		
Nod$_4$ - *gpgp*	81	33.5	18.0 ± 5.1
nod$_4$ *nod*$_4$ *Gp-*	92		
nod$_4$ *nod*$_4$ - *gpgp*	3		
	nod$_4$ - *cp*		
Nod$_4$ - *Cp-*	188		
Nod$_4$ - *cpcp*	77	24.6	16.6 ± 5.2
nod$_4$ *nod*$_4$ *Cp-*	82		
nod$_4$ *nod*$_4$ *cpcp*	2		
	gp - *cp*		
Gp - *Cp-*	244		
Gp - *cpcp*	11	178.4	10.4 ± 1.7
gpgp Cp-	26		
gpgp cpcp	68		

The linkage group determined for the gene *nod*$_4$ (chromosome 5) is confirmed by analysis of the F_2 populations from crossing K301 to the WL1132 and WL741 marked by the gene *gp*. In both hybrids, monohybrid segregation was observed for each of the genes in question, namely *nod*$_4$ and *gp*.

Table 3. Analysis of joint segregation in F_2 for *nod*$_4$ and the marker genes *gp* in crossing the mutant with the tester lines WL1232 and WL741.

Phenotype in F_2	Number of plants	χ^2	Recombination fraction
	Line 1132		
Nod$_4$ - *Gp-*	161		
Nod$_4$ - *gpgp*	88	26.7	25.0 ± 5.2
nod$_4$ *nod*$_4$ *Gp-*	62		
nod$_4$ *nod*$_4$ - *gpgp*	5		
	Line 741		
Nod$_4$ - *Gp-*	103		
Nod$_4$ - *gpgp*	53	14.9	27.9 ± 6.2
nod$_4$ *nod*$_4$ *Gp-*	51		
nod$_4$ *nod*$_4$ *gpgp*	5		

Thus, the gene *nod*$_4$ located in chromosome 5 is most likely mutant, the marker genes possibly forming the following sequence:

I ---------- 10.4 ± 1.7 --------- I ------------- 16.6 ± 5.2 ------------ I
Gp *Cp* *Nod*$_4$

Supernodulating plants of K301 were crossed with a range of pea cultivars for inducing new genotypes. In F_2 populations plants with the mutant phenotype were registered. On the basis of each of such plants, a line was developed. The resulting lines were traced to F_6 for stability. In F_7 the following characters were assessed: nodule number, nitrogenase activity, plant weight, bean and seed number per plant. The indices were compared with those for K301 itself and to the cultivars. Lines of genetic and breeding interest were developed from crosses of K301 with Torsdag. This cultivar has the following characteristics: abundant nodulation, high nitrogenase activity (higher than that of K301), nitrate tolerance. In Torsdag these characters are inherited together (pleiotropy) dominantly. A genetic analysis was performed at crosses of Torsdag to Ramonsky 77, which, contrast to the former, is typically noted for weaker nodulation, low nitrogenase activity, poor resistance to nitrates. F_1 plants exhibited traits of the cultivar Torsdag. F_2 plants displayed a Mendelian segregation ratio of 3:1 with Torsdag characters over Ramonsky 77.

Reciprocal graftings of Torsdag and Ramonsky 77 plants showed that such characters as numerous large nodules, high nitrogenase activity and resistance to nitrates in Torsdag are controlled by factors that form in the roots. Lines with the gene nod$_4$ (supernodulation and stem fasciation) and the gene controlling nodulation in the cultivar Torsdag have been isolated. Both little nodules, typical of K301, and large nodules, typical of Torsdag, form on these plants (Fig.2). Nitrogenase activity is high in these plants - even higher than that in Torsdag (Sidorova et al., 1994).

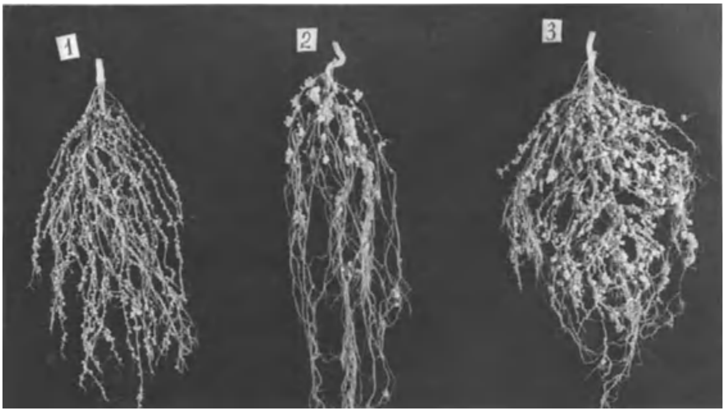

Fig.2. The root systems:
1. Supernodulating nts mutant K301.
2. Cultivar Torsdag.
3. F_7 hybrid K301 x Torsdag.

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POSITIONAL CLONING OF NODULATION GENES IN SOYBEAN: COUPLING DNA AMPLIFICATION AND BULKED SEGREGANT ANALYSIS

CAETANO-ANOLLÉS G., PADILLA J.E., GHASSEMI F., ABBITT S., KOLCHINSKY A., GRESSHOFF P.M.
Plant Molecular Genetics, Institute of Agriculture and Center for Legume Research, The University of Tennessee, Knoxville, TN 37901-1071, USA

In the positional cloning of genes involved in legume nodulation, we have generated markers linked to loci controlling nodule formation (*nts*, *rj1* and *rj6*) and early nodule development (*enod2*) in soybean (*Glycine max* L.) using bulked segregant analysis (BSA) (Michelmore et al., 1991) and genomic scanning with arbitrary oligonucleotide primers (Caetano-Anollés, 1994). Wild-type Bragg and EMS-induced mutants defective in autoregulation (nts382) or nodulation (nod49 and nod139) were crossed with *G. soja* PI468.397 to generate F2 and F3 segregating populations. DNA of individual segregants that expressed or failed to express a particular phenotypic (wild-type and mutant plants) or genotypic (RFLP pattern) trait were pooled and analyzed using DNA amplification fingerprinting (DAF) with sets of mini-hairpin (Caetano-Anollés and Gresshoff, 1994) and unstructured primers (Caetano-Anollés et al., 1991). The *enod2* gene was mapped on linkage group I of the Utah soybean map using Minsoy x Noir 1 recombinant inbred lines, and was linked to the seed coat color gene I and a quantitative trait loci (QTL) controlling race 3 cyst nematode resistance. BSA/DAF was used to find additional molecular markers in the region. Screening with 181 unstructured primers produced only one amplification marker placed 26 cM away from the supernodulation *nts* gene. The inefficiency to produce diagnostic markers indicates the relatively conserved nature of the *nts* region. In contrast, DAF with mini-hairpin primers and the generation of arbitrary signatures from amplification profiles (ASAP) (Caetano-Anollés and Gresshoff, submitted) were especially successful in detecting putatively linked polymorphisms. Some DNA polymorphisms were confirmed as bonafide markers linked to the trait of interest by individual analysis of segregants, and prepared to convertion or converted into sequence-characterized amplified regions (SCARs)(Paran and Michelmore, 1993). These sequence-tagged markers, together with others from a previous study (Caetano-Anollés et al., 1993), will be used for high density mapping of the relevant genomic regions and as landmarks for linking cloned soybean DNA from YAC and BAC libraries currently under construction.

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GENERATION OF YEAST ARTIFICIAL CHROMOSOME CLONES FROM SOYBEAN DNA AND THEIR USE IN PHYSICAL ANALYSIS OF THE GENOME BY FLUORESCENCE *IN SITU* HYBRIDIZATION.

FUNKE[1], R.P., ZHU[2], T., SHI[2], L., KEIM[2], P., LANDAU-ELLIS[1], D., AND P.M. GRESSHOFF[1].

(1) Department Plant Molecular Genetics and Center for Legume Research, The University of Tennessee, Knoxville, TN 37901-1071, USA.
(2) Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ 86011-5640, USA.

Yeast Artificial Chromosomes (YACs) containing soybean DNA were produced by ligating size selected, partially *Eco*RI digested chromosomal DNA to dephosphorylated YAC4 vector. Ligation mixtures were transformed into yeast spheroplasts (strain AB1380) and clones ranging from 50 to 900 kb (average size 175 kb) were recovered. About 10% of the soybean genome has been arrayed in clones stored in 96-well plates. About one third of the clones show very intense hybridization with labeled total soybean genomic DNA, indicating that they contain sequences that are represented many times in the genome. About one third of the clones show no signal at all, although they are clearly visible on ethidium bromide stained gels and hybridize to vector sequences. The rest of the clones show intermediate hybridization intensities. YACs in each of these classes were shown to be stable through repeated subculture.

Two low copy number clones and two clones showing intermediate hybridization were gel-purified and localized onto metaphase chromosome spreads of soybean by fluorescence *in situ* hybridization(FISH). Some YACs show two hybridization foci, indicating that they contain unique sequences. Others show a more complicated pattern consistent with the presence of segments of DNA that are duplicated in the genome. FISH showed that some YACs contain sequences that are sparsely scattered on all the soybean chromosomes, whereas others give very strong localized signals and probably contain tandemly amplified sequences that are specific to certain chromosomes. The development of these molecular tools should facilitate the positional cloning of symbiosis-related genes known only by their phenotype.

ANALYSIS OF SEQUENCE CHARACTERIZED AMPLIFIED REGIONS (SCAR) DERIVED FROM AFLP MARKERS LINKED TO THE SUPERNODULATION LOCUS OF SOYBEAN

PADILLA, J.E., CAETANO-ANOLLÉS, G., LANDAU-ELLIS, D., GRESSHOFF, P.M.
Plant Molecular Genetics, Institute of Agriculture and Center for Legume Research, The University of Tennessee, Knoxville, TN 37901-1071, U.S.A.

Genetic analysis of legume nodultion has contributed to define critical stages and hypothetical factors of the symbiotic interaction with *(Brady)rhizobium*. The nitrate-tolerant supernodulation (*nts*) locus is involved in the autoregulatory nodulation response of soybeans. This phenotype segregates as a recessive monogenic trait, is represented by allelic variants and appears to be conditioned by a shoot-derived factor (1). The locus is ≤ 1.0 cM distant from a well-described RFLP marker in one of the soybean linkage maps (2,3); by physical mapping estimations, it may be located within 500 Kb from this flanking marker (4). To isolate the *nts* gene by positional cloning, a set of tigthly-linked markers is required (1). In this work we focused on three complementary objectives:

a) Examine putative tecMAAP markers (5) to be used as SCARs linked to the *nts* region in soybean. Our analysis scheme showed that only a few of the previous markers fit into some of the predicted types. This survey also suggested that inconsistencies of some tecMAAP markers are generated by variation in the preparation of digested templates. Validation of SCAR requires a precise hybridization analysis and PCR amplification. These procedures must display the polymorphism to proceed with mapping and are not yet conclusive.

b) Standardize methods for rapid purification and cloning of AFLPs. By using iterative re-amplification and alternative techniques, most AFLP are recovered as inserts with defined structural features. Variation of the fast and reliable DAF-related procedures (mini-PAGE, silver-staining) have been optimized for clone characterization and sequencing.

c) Design alternative sampling strategies for BSA (see Caetano-Anollés et al., this proceedings) in order to focus on distinct map intervals for placing additional AFLP. By using segregating populations for supernodulation, we propose a set of pools displaying determinate molecular phenotypes (RFLP and SCAR). At least a loosely-linked marker was placed near the *nts* locus using this strategy (Kolchinsky, et al., unpublished).

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GENERATING DIPLOID NON-NODULATING ALFALFA MUTANT FROM A TETRAPLOID GERMPLASM.

ENDRE G., KALO P., CSANADI G., KISS P., FOLFOLDI K., KISS G.B.
Institute of genetics, Biological Research Center of the Hungarian Academy of Sciences P.O.BOX 521 Szeged H-6701 Hungary

Our main object is to isolate *Medicago* gene(s) playing role in the formation of symbiotic nodules by map-based cloning approach relying on the genetic map for diploid alfalfa constructed by our research group (Kiss et al.,1993). For this purpose we have chosen the Non-Nodulating MnNC-1008 (NN) *Medicago sativa* mutant. In this plant no root hair curling or cortical cell division can be induced that is a very early stage in the nodule organogenesis is impaired, so the mutated genes must have indispensable function in the nodulation process. Following the complex tetraploid inheritance of the two mutated genes, *nn1* and *nn2* (their contemporary presence is needed for the Nod- phenotype) in a tetraploid segregating population is excessively complicated. Therefore we decided to reduce the ploidity of MnNC-1008(NN) in order to make the mapping work easier. E.T.Bingham described a method (Bingham,1969) for generating diploids from tetraploi alfalfa with a cross between tetraploid and diploid plants. When tetraploid parent (which is male sterile or its pollens are removed before the crossing) is pollinated with the pollens of a diploid species four types of offsprings are produced: 1) self-pollinated tetraploids (in case of insufficient male-sterilization); 2) hybrid tetraploids coming from the cross; 3) hybrid triploids and 4) diploids containing the reduced genome of the tetraploid female parent. We tried to use this technique in order to get diploid mutant plant(s) containing *nn1* and *nn2*. The purple-flowered tetraploid MnNC-1008(NN) was crossed (after a male-sterilization treatment) with yellow flowered diploid *Medicago*. The accomplished approx. **7000** pollinations resulted in **72** seeds. From these 72 seeds 22 did not germinate, 24 germinated but the plantlets had low viability and **26** plants grown up was vigour and examined for their chromosome content. Among them there are mainly self-pollinated tetraploids and unstable hybrid tetraploids (mixoploids) and hybrid triploids, but **1** is a **diploid purple-flowered *Medicago*, called Dj5** inherited the Non-Nodulating phenotype. Having this diploid mutant plant we carried out crosses with a wild type diploid plant (*Medicago sativa ssp.quasifalcata* k93) to get F1 plant for producing the segregating population. Despite of flowering problems and low seed-producing ability of Dj5 we have got few seeds, and some of them germinated. Preliminary experiments support the hybrid nature of the progenies.

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A COMPARISON OF NODULATING AND NON-NODULATING AFRICAN SPECIES OF *ACACIA* USING MORPHOLOGICAL AND MOLECULAR MARKERS.

HARRIER, L.A., WHITTY, P.W., SUTHERLAND, J.M. AND SPRENT, J.I.
Dept. Biological Sciences, Univ. of Dundee, Dundee, DD2-4HN
Scotland UK

The multifaceted genus *Acacia* is one of the largest genera of vascular plants, comprising over 1200 species of which 140 are native to Africa. The vast majority of African *Acacia* species are thought to be able to form nodules and fix nitrogen in association with symbiotic bacteria. However there are 17 species which are thought to be unable to nodulate. Morphological analysis using 31 characters ranging from inflorescence type to seed morphology of 20 species, including 6 non-nodulating species was completed. Randomly amplified polymorphic DNA, using eight random oligonucleotide primers on the same 20 species plus a non-nodulating Madagascar species, producing 140 amplified products were used to analyze genetic diversity. The measure of similarity was calculated using Nei and Li's coefficient. Principal co-ordinate analysis and hierarchical cluster analysis was used to investigate grouping within the species. The non-nodulating species were found to form distinct groups apart from the other nodulating species examined and further subdivision of the nodulating species was achieved.

SYMBIOTIC MUTANTS AND NODULE-SPECIFIC ESTs OF THE MODEL LEGUME *LOTUS JAPONICUS.*

SZCZYGLOWSKI K., HAMBURGER D., KAPRANOV P., SHAW R.S., and DE BRUIJN F.J.
MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824, USA.

The aim of this research is to develop a model system to allow the genetic and molecular dissection of the plant processes underlying nodule formation and nitrogen fixation using the legume *Lotus japonicus* (Handberg, Stougaard 1992). In order to facilitate this goal, symbiotic variants of *L. japonicus* plants were generated using classical EMS mutagenesis. Approximately 30,000 M2 plants derived from 3000 M1 families were analyzed. Putative *L. japonicus* M2 mutants were further characterized in the M3 and the M4 generations, in order to establish heritability and genetic stability of the observed mutant phenotypes. Using this approach, twenty four stable *L. japonicus* symbiotic mutants were isolated. The phenotypes of these plant mutants can be classified into four basic categories: (a) no nodulation, [Nod$^-$]; (b) bushy roots and no nodulation, [Brl; Nod$^-$]; (c) ineffective white nodules or nodulation arrested at relatively early stage, [Nod$^+$; Fix$^-$], and (d) many more nodules produced compared to wild type, [Nod^{++}]. Genetic analysis of the identified mutants is now in progress to characterize the genetic determinants underlying the observed phenotypes. In addition, the construction of a nodule-specific expressed sequence tags (ESTs) library was initiated, using a recently developed method of RNA fingerprinting (Differential display; Liang, Pardee 1992). Eighty combinations of primers were used in this analysis and complex banding pattern reflecting changes in the population of hundreds of mRNAs at different stages of nodule development were displayed. Approximately 120 putative nodule specific cDNAs were isolated. A preliminary analysis of five cDNAs resulted in identification of one novel *lb* gene, and two novel nodule-specific genes sharing no similarity with any sequences in the DNA/protein databases. The results of these analyses will be presented and discussed.

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NODULATION, NITROGEN FIXATION, NITRATE ABSORPTION AND GROWTH OF A SUPERNODULATING SOYBEAN MUTANT EN6500 UNDER VARIOUS NITRATE LEVELS.

TAKAHASHI M. (1), KOKUBUN M. (1), AKAO S. (2).
(1) National Agriculture Research Center. Kannondai, Tsukuba, Ibaraki, 305 Japan.
(2) National Institute of Agrobiological Resources. Kannondai, Tsukuba, Ibaraki, 305 Japan.

Supernodulating soybean [*Glycine max* (L.) Merr.] mutants are considered to be a useful material for investigations of the soybean-*Rhizobium* symbiosis, especially host autoregulation mechanisms of nodulation. In 1992, a supernodulating mutant En6500 has been isolated from a major Japanese soybean cv. Enrei. In this study, we are to clarify the characteristics of nitrogen fixation, nitrate absorption and associated characters of En6500 at various nitrate levels in the medium, and to examine the reduced growth of En6500 compared to that of Enrei in relation to nitrate absorption.

The mutant En6500 and its wild-type parent Enrei were cultured hydroponically at various nitrate (KNO_3) levels (0.5, 5.0, and 15.0 mol m^{-3}) either under sterile conditions or inoculated conditions with *Bradyrhizobium japonicum*. For inoculated plants, ^{15}N labeled KNO_3 was used to determine the percentage of nitrogen derived from supplied nitrate. Plants were grown in a greenhouse with light supplement (6:00-20:00) under controlled temperature of 28°C/23°C (day/night) and then sampled at 33 days after sowing.

The mutant En6500 produced a larger number of nodules, higher nodule dry weight and a larger amount of fixed nitrogen per plant, thus depending more on fixed nitrogen at all nitrate levels than its parent cv. Enrei. The mutant also exhibited a nitrate tolerance in nodule number and nodule dry matter, though the amount of fixed nitrogen per plant or on a nodule dry weight basis was reduced with increasing nitrate levels. These results indicate that En6500 is more nitrate-tolerant in terms of nodule formation, but nitrogen fixation was particularly affected by nitrate.

The ability of nitrate absorption and plant growth in the mutant were less active than those of the parent even in the absence of inoculation. It is suggested that the suppression of nitrate absorption in En6500 was due to a smaller amount of root, rather than to a specific absorption ability, because the amount of nitrate absorption per g root in En6500 was greater than that in Enrei in the absence of inoculation.

Inoculation further reduced nitrate absorption and growth of the mutant. Root dry matter and nitrate absorption in En6500 decreased to half when plants were inoculated. Limited absorption sites of En6500 by the large number of nodules may result in the suppression of nitrate absorption. Shoot dry matter reduction by inoculation may not only be due to the direct effect of abundant nodules which consume carbohydrates for nodule formation and N_2 fixation, but also to the decrease of total nitrogen assimilation through the reduction of nitrate absorption.

ISOLATION OF NEW PEA *(PISUM SATIVUM L.)* SYMBIOTIC MUTANTS

TSYGANOV, V.E., BORISOV, A.Y., ROZOV, S.M.,
TIKHONOVICH, I.A.
All-Russia Research Institute for Agricultural Microbiology,
Podbelsky Chosse 3, St.Peterburg-Pushkin 8, 189620, Russia
Institute of Cytology and Genetics, Novosibirsk, 630090, Russia

Pea laboratory line SGE (Kosterin and Rozov, 1993) was used to isolate symbiotic mutants after experimental EMS-mutagenesis. Eight mutants were obtained. Two of them (SGEFix$^-$-1, SGEFix$^-$-2,) form ineffective root nodules, six (SGENod$^-$-1, SGENod$^-$-2, SGENod$^-$-3, SGENod$^-$-4, SGENod$^-$-5, SGENod$^-$-8) - are unable to form nodules at all.

The mutations of all above-listed mutants are recessive. The monogenic inheritance was shown for mutants SGEFix$^-$-1, SGEFix$^-$-2, SGENod$^-$-2, SGENod$^-$-3, SGENod$^-$-5, SGENod$^-$-8. The allelism tests revealed that mutants SGEFix$^-$-1 and SGEFix$^-$-2 are non-allelic to each other and to the mutant lines E135f (Kneen et al., 1990), FN1 (Postma et al., 1990) and Sprint-2Fix$^-$ (Borisov et al., 1992).

Contrary to the initial line SGE mutant SGEFix$^-$-1 forms numerous small white root nodules lacking nitrogenase activity. Mutant SGEFix$^-$-2 forms several types of nodules lacking nitrogenase activity: small and large white nodules with a dark pit on the distal end occur most often, pale-pink, pink and green nodules are less common. Study of the strain specificity of SGEFix$^-$-1 and SGEFix$^-$-2 mutants revealed no suppression of mutant phenotypes by any of the 10 strains of various geographic origin.

Thus, experimental mutagenesis is an appropriate approach for the identification of pea symbiotic genes and creation of genetic models of first level for molecular and morphological study of micro- and macrosymbiont interactions. Symbiotic mutants are the material for the construction of the second level genetic models, combining several mutations in one genotype.

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Kosterin OE Rozov SM (1993) Pisum Genetics 25, 27-31.
Postma JG et al (1990) Plant Sci. 68, 151-161.

ULTRASTRUCTURAL ANALYSIS OF NODULES IN TWO NEW PEA MUTANTS SGEFIX$^-$-1 AND SGEFIX$^-$-2

MORZHINA, E.V., TSYGANOV, V.E., BORISOV, A.Y.,
LEBSKY, V.K., TIKHONOVICH, I.A.
All-Russia Research Institute for Agricultural Microbiology, Podbelsky Chosse 3, St.Petersburg-Pushkin 8, 189620, Russia

Two EMS-generated ineffective SGEFix$^-$-1 and SGEFix$^-$-2 pea mutants were studied by transmission electron microscopy for comparing with the nodule ultrastructure of initial SGE pea line.

The mutant SGEFix$^-$-1 forms numerous small white nodules (1-2mm) that contain hypertrophied infection threads. The later are characterized by simultaneous destruction of their walls and matrix and by mass bacteria release from infection thread into host cytoplasm. Bacteroids increase several times and acquire a pleomorphic form, but the complete differentiation can not be achieved. Symbiosomes containing single bacteroid exist for a very short time, the adjacent symbiosomes fuse with the formation of aggregates.

The mutant SGEFix$^-$-2 forms nodules of various types. 1.The infected cells from the round small white nodules (1 mm) contain only infection threads from which bacteria can not release. 2.White nodules (1-3 mm) have a small pits on their distal ends and their histological nodule differentiation is disrupted. 3.The ultrastructure of elongated pinkish and greenish nodules (1-3 mm) is similar to one of the pea mutant Sprint-2Fix$^-$ (Borisov et al, 1992), but bacteroids are characterized by higher level of differentiation. The SGEFix$^-$-1 and SGEFix$^-$-2 mutants' nodules are characterized by early senescence in comparison with SGE line.

Obtained mutants demonstrate some morphological characteristics that make them different from other pea mutants (Sprint-2Fix$^-$, E135f, FN1) (Borisov, et al, 1992, Kneen et al, 1990, Postma et al, 1990).

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IDENTIFICATION OF THE EXACT FUNCTIONAL SEQUENCE OF THE TWO PEA *(PISUM SATIVUM L.)* SYMBIOTIC GENES USING THE GENETIC MODEL OF THE SECOND LEVEL.

BORISOV, A.Y., ROZOV, S.M., TSYGANOV, V.E., MORZHINA, E.V., LEBSKY, V.K., TIKHONOVICH, I.A.
All-Russia Research Institute for Agricultural Microbiology,
Podbelsky Chosse 3, St.Petersburg-Pushkin 8, 189620, Russia
Institute of Cytology and Genetics, Novosibirsk, 630090, Russia

To understand the plant genetic control of pea-*Rhizobium* symbiosis development it is important to identify the stage of the single symbiotic gene functioning. When studying the mutant phenotypes (the first level genetic models) separately it is difficult to point out exactly which gene is the first in the row of events blocking symbiosis development and leading to similar phenotypes. In this study we used mutants Sprint-2Fix$^-$ (mutation in gene symX (Borisov et al., 1992) and E135f (mutation in gene sym13 (Kneen et al., 1990). Earlier we have reported (Borisov et al.,1992) the results of comparative ultrastructural investigation of these pea Fix$^-$ mutant nodules. It has been preliminary suggested that the gene symX is expressed before the gene sym13. The line carrying mutations in these two loci (genetic model of the second level) was constructed to identify the exact sequence of their functioning. This line was named RBT. The plants of the line RBT form nodules on their roots which are similar to the line E135f both by morphology and number. But the ultrastructure of infected cells and symbiosomes of the line RBT is the same as that of line Sprint-2Fix$^-$. Consequently the mutation in locus symX suppresses manifestation of the mutation in locus sym13 in respect to bacteroid differentiation. Thus, gene symX functions at an earlier stage of symbiosis than gene sym13 but their manifestations are independent.

In this way, it was shown that the use of genetic models of the second level is a fruitful approach to identify the sequence of functioning of the single symbiotic genes controlling the close stages of symbiosis development. These models are also useful in the search for molecular markers of different symbiosis stages and in the search for the plant molecular signals controlling the symbiosis development

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MAPPING OF PEA *(PISUM SATIVUM L.)* GENES AFFECTING SYMBIOSIS

ROZOV, S.M., BORISOV, A.Y., TSYGANOV, V.E., MEN, A.E.,
TIKHONOVICH, I.A.
All-Russia Research Institute for Agricultural Microbiology, Podbelsky
Chosse 3, St.Petersburg-Pushkin 8, 189620, Russia
Institute of Cytology and Genetics, Novosibirsk, 630090, Russia

Analysis of inheritance of two groups of plant symbiotic genes (sym and nodulin genes) using morphological and molecular markers is convenient strategy for the mapping of these groups of genes, identification of their possible cluster organization and identity. This approach can provide data for further positional cloning of sym-genes.

We studied the inheritance of the genes symX (Borisov et al.,1992), sym7 (LaRue and Weeden,1992), and ENOD12 (Scheres et al.,1990) localized in linkage group 3. Data obtained from crosses of line Sprint-2Fix$^-$ (tester line for symX) were combined with the help of the computer mapping program JOINMAP (Stamm,1993). The resulting map of symX -region of pea linkage group 3 is shown below. Distances in this scheme are given in cM map units (Kosambi map function was used).

```
M          uni(tac)   symX                           st
-I-----------I----------I-----------------------------I-
0.0        14.1       31.7                          70.8
```

We also analyzed the inheritance of the other symbiotic genes (ENOD12 and sym7) which are localized in another region of this linkage group. The linkage between two gene pairs b-ENOD12 and b-sym7 was observed. The preliminary map for this region is shown below. More precised location of these loci in progress.

```
b     gl                Enod12              sym7
-I-----I---------------------I--------------------I-
0.0   2.6                 17.3                36.6
```

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Stamm P (1993) The Plant Journal 5, 739-744.

POSSIBLE REASONS OF INEFFICIENCY AND DEFECTIVE SYMBIOSOME DEVELOPMENT IN THE FIX⁻ PEA MUTANTS

CHVABAUSKENE J. A. (1), TCHETKOVA S. A. (1), SKOT L. (2), KRAVCHENKO L. V. (1), TIKHONOVICH I. A. (1) (1) Research Institute for Agricultural Microbiology, Podbelsky Shosse 3, St. Petersburg-Pushkin 8, 189620, Russia. (2) Institute of Grassland and Environmental Research, Plas Gogerddan, Aberystwith, Dyfed SY 23 3 EB, UK.

Plant mutants impaired in the late stages of symbiosis (bacteroid formation and function, bacteroid senescence) present a convenient model for the study of genetic control over symbiosome development. Comparative biochemical, physiological and cytological study of 3 Fix$^-$ pea mutants - E135f (Kneen et al, 1989), Sprint2Fix$^-$ (Borisov et al, 1992) and RBT, combining both mutations points out to the existence of at least two possible reasons of bacteroid non-differentiation and inefficiency. One is the defect in the flavonoid-like plant signals. The type of HPLC chromatographic profiles of flavonoid containing fraction from nodulated roots of all three mutants sharply differed from the parent one by the appearance of 4 pronounced peaks in the area of 13 - 33 min. Thus the character of flavonoid spectrum seems to correlate with the symbiotic status of the nodules - efficiency or inefficiency - and not with the genotype. However, the much larger peak at 26 min. in Sprint2Fix$^-$ may serve not only as a marker of inefficiency but also as a marker of a particular mutation.$^-$The nature and structure of these peaks is under study. The second possible reason of inefficiency lies in the change of carbon nutrition of bacteroids. The concentration of most important photosynthates in the nodules - sucrose and glucose - reflected the symbiotic status of the nodules being twice lower in the efficient parent cultivars than in the mutants. This fact reflects the much lower rate of carbon consumption connected with nitrogen fixation in the Fix$^-$ nodules in comparison with Fix$^+$ and serves as a marker of inefficiency. On the contrary the other sugar-like substance typical for the nodules - ononitol (Skot, Egsgaard, 1994) - correlated not with the Fix$^-$ phenotype but with the particular plant gene mutation. The mutation leading to the non-differentiation of bacteroids both alone in Sprint2Fix$^-$ and in the double mutant RBT lead to the drastic (10 times) decrease of ononitol content. Possible link between low ononitol concentration and the unusual pattern of JIM18 expression (Brewin, personal communication)in the nodules of this mutant is discussed.

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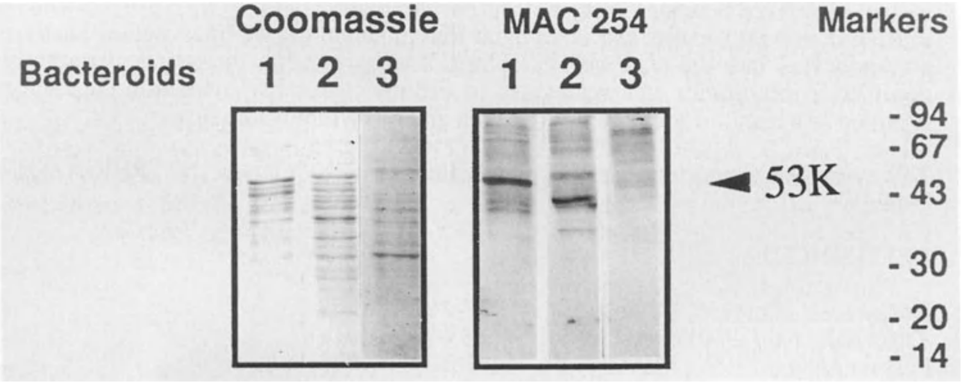
A GLYCOPROTEIN IN THE PERIPLASMIC SPACE OF PEA NODULE BACTEROIDS MAY BE OF PLANT ORIGIN

DAHIYA P.[1], VANDENBOSCH[2], TIKHONOVICH, I.[3], BREWIN, N.J.[1]
[1]John Innes Centre, Norwich NR4 7UH, UK;
[2]Dept. of Biology, Texas A&M Univ, USA;
[3]Institute for Agricultural Microbiology, St. Petersburg, Russia

The objective was to purify and characterise a 53 kD glycoprotein identified in pea nodule bacteroids by MAC 254. This monoclonal antibody reacts with a carbohydrate epitope on pea nodule lectin (N-lec1) and other members of a group of plant glycoproteins that are targetted to the peribacteroid fluid and peribacteroid membrane of pea nodule symbiosomes (Perotto et al. 1991). Therefore it is possible that the bacteroid-associated protein may also be of plant origin. Immunogold localisation studies indicated that the antigen accumulated in inclusion bodies in the periplasmic space of bacteroids. The glycoprotein was purified from isolated bacteroids by preparative gel electrophoresis, ConA-affinity chromatography and immunoaffinity chromatography on MAC254-sepharose. The purified material was subjected to N-terminal amino acid sequence analysis. By isolating bacteroids from symbiotically-defective nodules (Sprint2-Fix$^-$) it was found that with different host-*Rhizobium* combinations the bacteroids contained other glycoproteins that reacted with MAC 254 antibody. These components ranged in molecular weight from 65 - 20 kD. It is possible that they represent precursors and cleavage products related to the 53 kD component.



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MAY WE TALK ABOUT GENERAL MECHANISMS OF PLANT CONTROL OVER BACTERIA DIFFERENTIATION INTO BACTEROIDS?

LEBSKY, V.K., BORISOV, A.Y., TSYGANOV, V.E.,
MORZHINA, E.V., TIKHONOVICH, I.A.
All-Russia Research Institute for Agricultural
Microbiology, Podbelsky Chosse 3, St.Petersburg-Pushkin 8, 189620,
Russia

Using of a constantly growing set of pea *(Pisum sativum L.)* Fix$^-$ mutants together with such an informative method as electron microscopy allows to elucidate the main morphological abnormalities on several stages of the nodule development (infection threads formation; bacteria endocytosis; morphological differentiation of bacteroids; symbiosomes formation and nodule senescence) characterizing ineffective symbiosis. Such morphological studies allow to discuss general mechanisms of plant control over bacterium differentiation into bacteroids. Five non-allelic Fix$^-$ mutants were studied: two mutants: Sprint-2Fix$^-$ (Borisov et al., 1992), SGEFix$^-$-1, SGEFix$^-$-2 , E135f (Kneen et al., 1990) and FN1 (Postma et al., 1990). Summarizing our results and those obtained in other laboratories we can formulate some general principles describing the mechanisms of plant control over bacterial differentiation into bacteroids.

1. Plant genes controlling bacterial differentiation work in a certain sequence. However the sequence of switching on of these genes is far from being as strict as falling dominoes one after another. Their action may overlap one another.
2. Both in the infection thread and in the cytoplasm of the infected cells there exist several populations of bacterial cells with various levels of differentiation.
3. The host-plant when controlling symbiosome formation specifically adapts its system of defence reactions for symbiosis development.
4. Both in normal variants and in all plant Fix$^-$ mutants that we have studied bacteria are endocyted into the plant cell cytoplasm one by one. So formation of multiply occupied symbiosomes and aggregates in the infected cells' cytoplasm should be regarded as a result of bacterial proliferation and the symbiosome fusion.

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ISOLATION OF SYMBIOSIS-SPECIFIC PLANT mRNA FROM *TRIFOLIUM REPENS* ROOTS BY DDRT-PCR and SUBTRACTION HYBRIDIZATION-PCR

BJOURSON A.J. (1), COOPER J.E. (1), ORGAMBIDE G. (2) and DAZZO F. (2)
(1) Department of Applied Plant Science, The Queen's University of Belfast, Newforge Lane, Belfast BT9 5PX, Northern Ireland.
(2) Department of Microbiology, Michigan State University, East Lansing, MI 48824 USA.

Changes in plant root mRNA expression which are induced/repressed by rhizobia are highly localised and are difficult to isolate by traditional techniques. The main reason for this is that the responding mRNA from a few localised cells is obscured by the vast preponderance of other mRNA from non-responding cells. For example ENOD12 is expressed in only 3% of root hair cells (Zalenskii et al., 1992).

We have used differential display reverse transcription PCR (DDRT-PCR) and modified subtraction hybridization-PCR of root cDNAs to address this problem. DDRT-PCR analyses of uninoculated and *Rhizobium trifolii* (ANU843)-inoculated roots resulted in the isolation of 65 putative uniquely/differentially induced/repressed cDNA sequences. Thirty of the 65 cDNAs have been cloned and partially sequenced. Two of these cDNAs exhibit significant sequence homologies with previously isolated plant genes. DD12 (547 bp) is homologous to *M. truncatula* ENOD40 (83%) and DD22a (336 bp) is homologous to plant phosphoglycerate mutase (75%). A third clone, DD23b (336 bp), exhibited limited amino-acid sequence homology to regions of phospholipase A2 (involved in signal transduction, specifically in the oxidative burst in neutrophils) and ENOD14. RT-PCR expression analyses demonstrated that DD23b mRNA is uniquely induced by *R. trifolii* within 24h after inoculation. *R. trifolii* chitinolipooligosaccharide (CLOS) treatment of roots also specifically induced DD23b in white clover.

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DIVERSITY OF ORIGINS AND EXPANSIVE MODES OF PBM AND MORPHOLOGICAL RELATIONSHIP BETWEEN BACTEROIDS AND HOST CELLS IN LEGUME NODULES

SHAN XUEQIN and JING YUXIANG
Institute of Botany, Chinese Academy of Sciences, Beijing
100044, China

Bacteroids in legume nodules are enclosed in peribacteroid membranes (PBMs). There are more than two or three, or even more bacteroids in one PBM of the nodules of Sesbania cannabina, Glycine max and Pisum. We observed that in the formation of PBMs and the development of bacteroids which existed from one to more numbers in one PBM, the origins of membranes on a large scale and expansion of the PBM could probably meet the requirement for symbiotic physiology. It was showed that in the same nodule sample there were several models of the PBM expansion, such as chimeric fusion between, or among the PBMs which possessed protrusions and hollows, fusion of the PBM with endoplasmic reticulum(ER) and its vesicles, with small vacuole membrane etc. Cytochemistry indicated that the PBM, plasma membrane etc. had the same type of ATP-ase, and the PBM had the structural properties similar to the plasma membrane. Therefore, the PBM appears to be a mosaic membrane possessing features of the plasma membrane, ER membrane, and vacuole membrane. Finally, it was discussed that only the diversity in the PBM origins could make a great number of the rhizobia, which were released on a large scale, on the one hand, from infection threads, on the other hand, devided rapidly in host cell cytoplasm, be enclosed, and could meet the requirement for more membranes in the stage of the PBM expansion of the nodule development. After the development from rhizobia of the bacteroids, although which are enclosed in the PBMs originated from the host cells, substance exchange between the bacteroids and the host cells are conducted at any time not only at the physiological and biochemical levels, but also are displayed in the morphological structure. It was observed that mutual protrusions of both the PBMs and the surface of bacteroids existed, and some of them touched and fused together to form a pipe-like canal structure in the early stage, or mature stage of Chinese milkvetch (Astragalus sinicus) nodule development. We consider that this structure is an active embody of symbiotic action and provides the morphological evidence for the substance exchange between the bacteroids and the host cells.

TRANSGENIC CLOVER WITH PROMOTER:*GUS* FUSIONS: TOOLS TO STUDY CLOVER ROOT AND NODULE DEVELOPMENT.

DJORDJEVIC, M.A., MATHESIUS, U., LARKIN, P.* GARTNER, E.,
HALL, E., WEINMANN, J.J. AND ROLFE, B.G.

Plant Microbe Interaction Group, R.S.B.S, Australian National University, Canberra, 2601 Australia. *CSIRO Division of Plant Industry, Canberra, Australia.

We have examined the clover-*Rhizobium* interaction using transgenic subterranean and white clover. White clover were transformed at high efficiency using *Agrobacterium*-mediated transformation (Larkin P.J. et al. submitted). Translational fusions of the GUS gene to the clover *CHS1*, *2* and *3* promoters or the auxin responsive *GH3* promoter from soybean (Hagen et al, 1984) were generated to examine the role in root development and root nodulation of (a) the phenylpropanoid pathway and (b) the hormone auxin. *GUS* expression patterns in plants containing the *CHS1, 2,* or *3* promoter indicated that these genes were developmentally regulated during root and nodule formation, being expressed in the meristematic tissues and xylem parenchyma of the vascular traces. *CHS1, 2* and *3* were not expressed early in the infection process. Consistent with the hypothesis that these promoters respond to developmental signals, the *CHS1* promoter responds to auxin (Djordjevic M.A. et al submitted).

GH3:GUS responded to the addition of auxin but no other class of phytohormone. *GUS* activity in untreated plants was high during plant responses known to be influenced by auxin: lateral root initiation and gravi-stimulation (Evans, 1991). *GH3:GUS* expressed consistently in the vascular tissue of roots. Bursts of *GH3:GUS* expression were found to be strictly localised to points in the root cortex adjacent to initiating lateral roots. Emerging lateral roots grow directly and accurately through these spots of *GH3:GUS* activity. Outer cortical cells did not show *GH3:GUS* during root nodule formation. All root meristems, including those of nodules, show inconsistent activity during growth. During gravi-stimulation, a rapid induction of *GH3:GUS* activity occurs within 1 h on the non-elongating side of the root, near the fulcrum of root curvature, indicating that changes in auxin concentration or auxin sensitivity were occurring. Although *GH3:GUS* activity is inconsistent in meristem tissues, our results indicate that auxin is required to initiate lateral root and nodule meristems and that GUS activity in plants containing *GH3:GUS* will reflect auxin-mediated processes.

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THE STRUCTURE OF NODULATED CLOVER ROOTS INFECTED WITH MYCORRHIZA

JAMES E.K., DAFT M.J., JAMES W.M.
Department of Biological Sciences, University of Dundee, Dundee
DD1 4HN, UK.

Seedlings of the clover species *Trifolium repens* and *T.pratense*, were inoculated with *Rhizobium leguminosarum* bv. *trifolii* strain RCR221. At 20 d after rhizobial inoculation half the plants were inoculated by spores of *Glomus etunicatum*. By 54 d after mycorrhizal inoculation (54 DAI) the dry weight of mycorrhizal plants was significantly greater than non-mycorrhizal controls. Harvests prior to this revealed that the growth of nodules on the mycorrhizal-inoculated plants was increased and many had reached the mature, nitrogen-fixing stage at 14 DAI *ie* up to 12 d before the non-mycorrhizal controls. At this stage, there were spores attached to roots, numerous vesicles in nodulated roots and sparse hyphae on young nodules. By 40 DAI roots and nodules had a profuse covering of hyphae with germinated spores attached to roots adjacent to mature, N_2-fixing nodules. Arbuscules were well developed in regions of roots close to nodules and these regions were clearly involved in mycorrhizal nutrient uptake and exchange with the host plant. Also within these roots there was a large number of intercellular hyphae, with many of the hyphae originating from vesicles formed at the base of nodules. In some nodules on red clover, hyphae were seen in senescing infected cells at the nodule base. These infections did not appear to have a negative effect on the nodules as the latter retained meristematic activity and numerous healthy, bacteroid-containing cells. However, at no point in this study were mycorrhizas seen within the active region of the infected zone. It is probable that the low pO_2 within the nodule inner cortex is inhibitory to the spread of mycorrhizae, although some hyphae were seen in the outer cortex. The close proximity of vesicles and arbuscules to (and in) the base of the nodules suggests that nodules and mycorrhizae may benefit from a direct exchange of nutrients (eg. fixed N for P) rather than indirectly, via cycling of nutrients through the host plant.

REGULATION OF NODULATION IN *Alnus incana - Frankia* SYMBIOSIS.

WALL, L. G. (1) and HUSS-DANELL, K. (1, 2)
(1) Dept. of Plant Physiology, Umeå Universtity, S901 87 Umeå, Sweden.
(2) Dept. of Crop Science, Swedish University of Agricultural Sciences,
Box 4097, S904 03 Umeå, Sweden.

Actinorhizal plants form root nodule symbioses with N_2-fixing actinomycetes, *Frankia*. Only compatible strains of *Frankia* can infect *Alnus* roots. An early sign of the interaction is root hair deformation. Root nodules are often found as clusters along a root. This observation indicates some kind of regulation in nodule development when infective *Frankia* units are in surplus in the rhizosphere. In *Alnus Frankia* infects compatible host roots through deformed root hairs. *Frankia* hyphae penetrate at the site of folding and grows towards the interior of the tissue always surrounded by host cell wall deposition. Simultaneously, increased mitotic activity occurs in the root cortex in proximity to infected root hairs. Some of these dividing cells become infected by the growing *Frankia* hyphae. Meanwhile, the nodule meristem start to develop as a secondary root from the pericycle. When this new tissue reaches the prenodule region, the emerging nodule become infected, and central vascular bundles develop.
We have studied regulation of nodulation in *Alnus incana* (L.) Moench using growth in plastic pouches and a slide technique to observe root hair deformation. A crushed nodule suspension of "the local source of *Frankia*" (ls*F*) was used as inoculum (250 µg of nodules/plant). In a time series of double inoculation experiments, using split root system in growth pouches, we have observed systemic inhibition of nodulation starting after 3 days from inoculation. In another experiment different *Frankia* isolates were inoculated in the first side: CpI1, ArI3, an isolate from the *lsF*, CcI3, R43 and the ls*F*. Four weeks later the second side was inoculated with the ls*F* . No inhibitory effect on nodulation by the *lsF* in the second side was observed after first inoculation with CcI3 or R43 (isolates from *Casuarina*, non-infective in *A. incana*). From double inoculation experiments with intact roots and nodule excision before the second inoculation we conclude that the regulation of nodulation involves at least two different mechanisms: (a) arrest of infections previous to nodule emergence. (b) loss of susceptibility in the elongating root tip of growing roots. The pattern of nodule distribution appears quite constant between main and lateral roots independently of the concentration of inoculum. We have found that susceptible root zones for infection shift from the main root to lateral roots after the first infections have occurred. A very crowded and short root hair deformation was observed in the growing root, only after the first infections had occurred. We interprete this effect as a plant defence reaction that prevents further infections to happen as part of the regulatory mechanisms. Thus, nodulation in *Alnus incana-Frankia* symbiosis shows a complex systemic regulation, that share some features with regulation in legumes.

ULTRASTRUCTURE OF MITOCHONDRIA IN LEGUME ROOT NODULES

ANDREEVA, I.N., KOZHARINOVA, G.M.
Laboratory of Nitrogen Metabolism, Timiryazev Institute of Plant Physiology, Russian Academy of Sciences, Moscow, 127276, Russia

The formation of symbiotic system in nodules of legumes is accompanied by structural and functional reorganization of both pro- and eukaryotic cells. However, the activity of mitochondria is partly inhibited by oxygen dificiency in the infected cells. So, during symbiogenesis, some adaptive modification appeared which permitted the mitochondria to maintain their activity. We studied the mitochondria of infected and uninfected cells both in effective and ineffective root nodules of broad bean, yellow lupine and common sainfoin. By using morphometric computer-assisted analysis morphological description of mitochondria in infected and uninfected intact cells (their section areas, shape, and the number of cristae) were compared. In the effective root nodules of bean, 30-40% of mitochondria in infected cells were significantly larger and contained more cristae than in uninfected cells. The enlargement of mitochondria volume did not affect the ultrastructure of mitochondria, no mitochondria swelling, or destruction occured. This proves that a new population of larger and functionally active mitochondria appeared after plant cell infection. However, inoculation with ineffective strain of rhizobia did not cause any change in the ultrastructure of mitochondria in infected cells. Effective symbiosis is accompanied by an enlargement of the total area occupied by mitochondria at the cell section in yellow lupine and common sainfoin. The total mitochondrial area was 1,4-2,5 times more in effective than in ineffective nodules. In the infected cell, most mitochondria were located at the periphery, under the cell wall, especially near intercellular spaces. Apparently, mitochondria intercept oxygen which diffuses from intercellular spaces through a cell wall. Increased mitochondria volume and number of cristae in infected nodule cells as well as the minimized operation of alternative, less efficient respiration pathway (Rawsthorne, La Rue 1986) appear to be adaptive responses for overcoming oxygen limitation. Another important function of these organelles - interception of oxygen entering the cell, which provides for defending nitrogenase against surplus of oxygen.

Rawsthorne S, La Rue TA (1986) Plant Physiol. 81, 1097-1102.

THE PECULIARITIES OF NITROGEN-FIXING SYMBIOSIS OF VETCH (*VICIA VILLOSA ROTH*) MUTANTS.

GALAN, M., LISOVA, N.
Research Institute of Agriculture and Cattle-Breeding West Region
UAAN, Lviv-Obroshyn, Ukraine

Using induced mutagenesis we obtained 4 mutant forms of hairy vetch (cultivar Stavchanka) with altered stem and leaf phenotypes and also tetraploid form. Morphology and symbiotic properties of these mutants were studied.

Mutant plants Vusata were characterized by the total reduction of leaves to the tendrile during flowering.

Mutant plants with fascilated and thickened stem have an axial type of floscule position, partial growth determination of the main stem and shoots of second order. They have good productivity. The form with wide leaves had an extended area of leaves and, correspondingly, high green mass.

Tetraploid mutant was characterized by increased sizes of all organs and possesses high potential of fodder productivity. Plant cultivar Shyrokolysta have enlargely of leaves surface.

Inoculation of vetch mutant forms with strains R.leguminosarum increase their yield crops of green mass in 13-19 per cent. Most production symbiosis provided for cultivar Stavchanka and Tetraploid form.

Studied of vetch forms by symbiotic parameters showed that cultivar Stavchanka and their Tetraploid form to a marked degree higher by nitrogenase activity as other mutants.

By many investigation and on variety legume crops showed, that production of symbiosis in some sort is formed by plant genotypes and microsymbionts and their interaction.

We studied of the cultivar-strains peculiarities of five forms vetch and six strains rhizobia.

It is established that level of production green mass of vetch is formed both the plant genotype and the strain genotype.

Two-factor dispersion analysis of the experiment results showed that the crops essentially were determined by genotype of the vetch forms with the level of effect 24 per cent; of the strain genotype - 0,8% and of cultivar-strains interaction - 6,1%.

DEVELOPMENT OF SAINFOIN (*ONOBRYCHIS VICIEAFOLIA*) ROOT NODULES AND ITS RELATION WITH NITROGENASE ACTIVITY

SHANHUA H.
Department of Biology, Sichuan Normal University,
Chengdu 610066, China

Development of sainfoin root nodules was studied. Their nodules were formed 18 days after inoculation of seedlings with rhizobium strain R$_2$, usually are spherical or cylindrial, then their front tips branch, gradually become to ginger-shaped. The active symbiotic regions of the root nodules are quite small in size, there only are a small guantity of infected cells in them. The infection threads are very abundant, they appear not only in young and mature infected cells, but also in senescent infected cells. The infection threads are smaller, their walls often hollow, even can divide their infection thread matrix to some obvious different parts. An infection thread generally contains a few bacteria which do not have polyphosphate granule, sometimes although they possess poly-β -hydroxybutyrate (PHB) granule, every bacterium usually has 1-2. After the bacteria are released to their host cytoplasm, their PHB granules disappear immediately (Han et al., 1995) . Every young bacteroid possesses a peribacteroid membrane, but when they develop to mature, the phenomenon, many bacteroids are surrounded by a peribacteroid membrane is frequently found (Han, 1990) . Following bacteroidal senescence, bacteroidal stain becomes dark and their intrastructures gradually become into vesicular-shaped and membrane-shaped up to complete disintegration.

Nitrogenase activity of sainfoin root nodules is relation with their development. The nitrogenase activity in early stage is very low, then gradually increase, when the root nodules develop to mature, their nitrogenase activity reaches to the highest, is about 18.6umolC_2H_4/g (fresh weight) . h.

Han SH et al. (1995) Acta Microbiologica Sinica. 35, 000-000.
Han SH. (1990) Acta Botanica Sinica. 32, 809-911

ELECTRON MICROSCOPY OF UNINFECTED AND INFECTED CELLS IN *ARACHIS HYPOGAEA* L. ROOT NODULES.

TANDON SHIV R.
Department of Biology, University of Wisconsin-Platteville
Platteville, Wisconsin 53818 USA

INTRODUCTION: *Arachis hypogaea* L. root nodules infected with rhizobia reveal a compact central bacteroidal tissue surrounded by a multilayered cortex without a scleroid layer. The bacteroidal tissue consists primarily of infected cells and a "few" interspersed rows of uninfected cells. This paper describes the similarities and differences between the two types of cells as they differentiate and specialize to play a vital role in nitrogen fixation in peanut root nodules.

RESULTS: Early in the infection process and throughout the nodule development both the infected and uninfected cells enlarge but remain of similar size. They contain dense cytoplasm; numerous mitochondria, dictyosomes, and ribosomes; vacuole(s); and endoplasmic reticulum. The plastids and mitochondria do not congregate next to the cell membrane in either type of cells. Although these similarities are obvious, the two types of cells reveal a marked ultrastructural difference:

Infected Cells	Uninfected Cells
a. Rod shaped bacteria which develop into spherical bacteroids	a. None present
b. Few, but small peroxisomes which do not enlarge during nodule development	b. Numerous peroxisomes which enlarge during nodule development
c. Few plastids	c. Numerous plastids with massive starch reserve
d. 1 to 2 unbounded bodies in a single profile	d. Unbounded bodies are not seen
e. 1 to 2 unidentified material in a single profile	e. Unidentified material has not been observed

DISCUSSION: The unbounded bodies observed in peanut have also been seen in the infected cells of soybean nodules. The unidentified material has been reported here for the first time. The role of both these structures in the nodule physiology has not yet been elucidated.

REGULATION AND FUNCTION OF THE *SESBANIA ROSTRATA* EARLY NODULIN GENE *ENOD2*

CHEN R.[1,2] and DE BRUIJN F.J.[1,3]
MSU-DOE Plant Research Laboratory[1], Department of Biochemistry[2] and Department of Microbiology[3], Michigan State University, East Lansing, MI 48824, USA

Enod2 is a nodulin which is expressed during the early stage of nodule development and has been postulated to be a cell wall protein. *In-situ* localization studies have shown that the *Enod2* gene is expressed in a specific cell layer in the nodule called the parenchyma, which is surrounding the central infected nitrogen-fixing zone (van de Wiel et al., 1990). The parenchyma cell layer has been suggested to be an oxygen diffusion barrier, but this hypothesis has not been directly tested thus far (Tjepkema, Yocum, 1974; Nap, Bisseling, 1990). We are interested in understanding the molecular mechanisms regulating the tissue-specific expression of the *Enod2* gene during nodule development and elucidating the possible role the Enod2 protein may play in nodule function (oxygen diffusion barrier formation). We have used the tropical legume *Sesbania rostrata* and its symbiont *Azorhizobium caulinodans ORS571* as system for our analysis (Dehio, de Bruijn, 1992). We have introduced a variety of chimeric *Enod2-gus* reporter gene fusions, containing sequences from the *Enod2* 5' and/or 3' regions, into *Lotus corniculatus* plant. Transgenic plants were nodulated and tissues analyzed by GUS histochemistry and activity quantification. Our data show that the 3' region of the *SrEnod2* gene is essential for its expression in the nodule parenchyma cell layer, since substitution of the 3' region of the *SrEnod2* gene with the 3' region of the nopaline synthase leads to very low or non detectable GUS activity. GUS activity directed by the construct carrying both the *Enod2* 5' and 3' regions flanking the *gus* reporter gene was localized in the nodule parenchyma cell layers, as well as the vascular tissue transversing the parenchyma. To define further the role of the 3' and 5' region of the *SrEnod2* gene in tissue-specific expression, we have made deletions in both the 5' region and the 3' region of the gene. We are currently regenerating and analyzing transgenic plants harboring these chimeric constructs. To study the function of the Enod2 protein, we have made antisense *Enod2* constructs and introduced them into *L. corniculatus*. Preliminary results obtained from the phenotypic and physiological analysis of transgenic plants harboring these antisense *Enod2* constructs will be presented.

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USE OF DIFFERENTIAL DISPLAY TO IDENTIFY NOVEL *SESBANIA ROSTRATA* GENES ENHANCED BY *AZORHIZOBIUM CAULINODANS* INFECTION

GOORMACHTIG S. (1), VALERIO-LEPINIEC M. (1), VAN MONTAGU M. (1), DE BRUIJN F.J. (2,3), HOLSTERS M. (1)
(1) Laboratorium voor Genetica, Universiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium; (2) MSU-DOE Plant Research Laboratory and (3) Department of Microbiology, Michigan State University, East Lansing MI 48824, USA.

Upon infection with *Azorhizobium caulinodans* ORS571, nodules are formed on the roots as well as on the stems of the tropical legume *Sesbania rostrata*. Stem nodules appear at multiple predetermined sites consisting of dormant root primordia, which are positioned in vertical rows along the stem of the plant. Profuse, site-specific and synchronous nodule formation makes the *S. rostrata* stem nodulation system uniquely suitable for studies on temporal expression patterns of plant genes specifically induced or enhanced during nodule ontogeny (nodulin genes). We used this system and the differential display method to isolate and characterize three cDNA clones (didi-2, didi-13, and didi-20; DIfferential DIsplay), corresponding to novel genes the expression of which is specifically induced or enhanced after rhizobial infection. DNA sequence analysis of these partial cDNA clones and subsequent database searches revealed that the deduced didi-2 gene product shares significant similarity with hydroxyproline-rich cell wall proteins. The didi-13 and didi-20 products are similar to chitinases and chalcone reductases, respectively. These studies show that differential display is a useful method for the isolation of novel nodulin genes.

IS THE EARLY NODULIN ENOD2 ASSOCIATED WITH REGULATION OF NODULE PERMEABILITY TO OXYGEN?

HUNT S.[1], WYCOFF K.L.[2], LAYZELL D.B.[1], HIRSCH A.M.[2]
[1]Department of Biology, Queen's University, Kingston, Ontario, Canada K7L 3N6. [2]Department of Biology, University of California, Los Angeles, CA 90024, USA.

The ENOD2 protein is expressed specifically in the inner cortex of legume nodules and its location coincides with the site of a putative barrier to O_2 diffusion. This has caused speculation that ENOD2 may be involved in the regulation of nodule permeability to O_2. Evidence has also been presented that an extracellular glycoprotein, MAC236, has a role in diffusion barrier regulation. If so, the levels of ENOD2 and MAC236 in nodules may be expected to differ in nodules with different permeabilities to O_2. This hypothesis was tested by measuring the levels of ENOD2 and MAC236 in nodules of alfalfa which developed during continuous exposure of roots to 8, 20 or 50% O_2. The roots were grown under these O_2 regimes for 20 d after inoculation with *Rhizobium meliloti* 1021, and then nitrogenase activities (H_2 evolution) were measured at the growth pO_2. In another set of plants, nitrogenase activity was measured at the growth pO_2, and then while pO_2 was increased gradually to 100%. In both sets of plants, nodules were harvested into liquid N_2 immediately after completion of the nitrogenase activity measurements, and analyses of ENOD2 and MAC236 protein were made by ELISA. Abundance of ENOD2 mRNA was assessed by Northern Blot analysis.

In all O_2 regimes, high nitrogenase activities were measured at the growth pO_2, indicating that the nodules had similar infected cell O_2 concentrations. Nodule permeability would therefore be greatest in nodules grown at 8% O_2 and lowest in nodules grown at 50% O_2. High nitrogenase activities were maintained as pO_2 was increased from growth levels to 100% O_2, indicating that nodules in all treatments were able to regulate their permeability to O_2. Levels of ENOD2 protein and mRNA did not differ among the nodules subjected to different growth conditions, suggesting that ENOD2 is not involved in the long term regulation of nodule permeability. The MAC236 glycoprotein was 2.4 times more abundant in nodules grown at 20% O_2 compared to those grown at 8% O_2, but its level was similar in nodules grown at 20 and 50% O_2. The evidence for a role of MAC236 in long term diffusion barrier regulation is, therefore, inconclusive. Although nodules from all treatments were able to reduce their permeability to O_2 as pO_2 was increased to 100%, the levels of ENOD2 and MAC236 proteins measured at 100% O_2 were similar to those measured at the growth pO_2. This indicated that neither protein is involved in short term regulation of diffusion barrier permeability.

The experiments above were repeated using transgenic alfalfa plants with reduced levels of ENOD2 mRNA and protein. No differences were observed between the transgenic and wild type populations in their ability to regulate nodule permeability. This provide further evidence that ENOD2 is not involved in diffusion barrier regulation.

THE PROMOTER OF THE *Vicia faba* L. VFENOD-GRP3 GENE ENCODING A GLYCINE-RICH EARLY NODULIN MEDIATES A PREDOMINANT GENE EXPRESSION IN THE INTERZONE II-III REGION OF TRANSGENIC *Vicia hirsuta* ROOT NODULES

KÜSTER H (1), SCHRÖDER G (1), FRÜHLING M (1), PICH U (2), RIEPING R (1), QUANDT H-J (1), BROER I (1), SCHUBERT I (2), PERLICK AM (1), PÜHLER A (1)
(1) Universität Bielefeld, Lehrstuhl für Genetik, Postfach 100131, D-33501 Bielefeld, Germany, (2) IPK, Abteilung Zytogenetik, Corrensstraße 3, D-06466 Gatersleben, Germany

A nodule-specific transcript sequence from a broad bean nodule cDNA library was expressed predominantly in the interzone II-III region of *V. faba* root nodules. Since time course experiments revealed an early expression in the root nodule, this cDNA encoded an early nodulin. The deduced amino acid sequence was characterized by a signal peptide and a C-terminal domain exhibiting a high content of glycine. Therefore, the cDNA was designated VfENOD-GRP3. As reported for other plant glycine-rich proteins, the ENOD-GRP3 protein might be located in the cell wall. Sequence analysis of a genomic clone revealed that the signal peptide and the glycine-rich domain were specified by two separate exons. Southern blot experiments indicated the presence of a VfENOD-GRP3 multigene family. By PCR experiments using sequence specific primers and DNA of microisolated broad bean chromosomes as a template, this gene family was located on the long arm of *V. faba* chromosome I. Primer extension identified two adjacent transcription start sites for VfENOD-GRP3 transcripts, which are possibly derived from different isogenes. The common nodulin sequences "AAAGAT" and "CTCTT" were located five and three times on both DNA strands of the putative VfENOD-GRP3 promoter region, respectively. In addition, three sequence motifs resembling organ-specific elements of the soybean *lbc*3 gene promoter and a sequence similar to the binding site 1 for the nodule *trans*-acting factor Nat2 were identified (Küster et al., 1995a). A fusion of the -1252/+10 promoter to a *gus*Aint reporter gene revealed a predominant GUS expression in the interzone II-III region of transgenic *Vicia hirsuta* nodules. In addition, a reduced GUS expression in the adjacent cell layers of the prefixing zone II and patches of weak GUS expression in the nitrogen fixing zone III were detected. This expression corresponded to tissue print data obtained for VfENOD-GRP3 gene expression in broad bean nodules. A *gus*Aint fusion of the -737/+10 promoter region resulted in a GUS expression pattern comparable to that mediated by the full-length promoter, whereas a fusion of the -239/+10 promoter devoid of sequence motifs resembling nodulin gene promoters mediated a reduced GUS expression in the interzone II-III region. We conclude that novel regulatory elements mediating a predominant VfENOD-GRP3 gene expression in the interzone II-III region are present on the -239/+10 promoter and that promoter elements from the -737/-240 and the -239/+10 promoter regions are interdependent for a maximal interzone II-III predominant gene expression (Küster et al., 1995b).

Küster H et al. (1995a) Plant Mol Biol, in press.
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LECTIN GENE EXPRESSION IN PEANUT NODULES.

LAW I.J.
Agricultural Research Council, Plant Protection Research
Institute, Private Bag X134, Pretoria 0001, South Africa

Peanut (*Arachis hypogaea* L.) is unique amongst legumes studied in containing nodule lectins. While certain legume lectins have been implicated in the recognition of infective rhizobia, the peanut nodule lectins may function, *inter alia*, in protein storage/packaging and may contribute to the efficient performance of peanut nodules (Law, Van Tonder, 1992). The galactose-binding lectin (GL) in nodules was previously found to differ in certain properties from peanut seed GL. The present study provided evidence that the individual lectins are produced by different genes and examined GL expression during nodule development.

GL-encoding cDNA clones were isolated using cDNA libraries from the seed and nodules of peanut. Sequence analysis indicated that clones from seed corresponded closely to a selected clone, SB2, whereas nodules yielded two different clones NG24 and NG30. Nucleotide and amino acid homologies between the lectin-encoding regions of SB2 and NG30 were relatively high (92.0 and 89.2 %, respectively) whereas those between SB2 and NG24 were lower (78.1 and 72.3 %, respectively). Clone NG30 contained a glycosylation site that was absent in clones NG24 and NG30.

Peanut nodules emerged 10 d after inoculation but NG24 and NG30 expression was first detected at 14 d when nodules turned pink. This indicates that neither clone is involved in rhizobial recognition. Maximum expression ocurred at 28 d. Levels of NG24 were higher and declined more slowly than those of NG30 over 42 d. Nodule GL is probably not a nodulin as NG24 was detected in roots of inoculated plants at 21-28 d. However, expression appears to be governed by nodule-related processes as neither clone was detected in roots and shoots of uninoculated N-control plants, or shoots of inoculated plants. Their late expression indicates that NG24 and NG30 are not involved in rhizobial recognition.

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INDUCIBLE EXPRESSION OF ANTISENSE ASPARTATE AMINOTRANSFERASE-P_2 IN *LOTUS CORNICULATUS* NODULES.

METT V, PODIVINSKY E, TENNANT AM, LOCHHEAD LP, JONES WT, REYNOLDS PHS.
Horticulture and Food Research Institute, Palmerston North, New Zealand.

A vector system, based on copper controllable gene expression (Mett et al., 1993), has been developed to give control over place (spatial) as well as time of expression (temporal) of an introduced gene. The system consists of two elements: (i) the yeast *ace*1 gene encoding a metallo-regulatory transcription factor, ACE1, under control of either an organ-specific or a constitutive promoter; and (ii) a gene of interest under control of a chimeric promoter consisting of the 46bp TATA fragment of the CaMV 35S RNA promoter linked to four repeats of the ACE1 binding site. The functioning of the system was demonstrated in "wild-type tops/transgenic roots" *Lotus corniculatus* using the GUS reporter gene. After addition of copper ions to the plant nutrient solution, GUS expression was visualised either specifically in nodules or in both roots and nodules when the *ace*1 gene was placed under control of the *nod*45 promoter or the CaMV 35S RNA promoter, respectively.

The nodule specific system was used to express antisense constructs of aspartate aminotransferase-P_2 (AAT-P_2)in *Lotus corniculatus* plants. AAT-P_2 is one of the two isoenzymic forms of AAT found in the plant fraction of leguminous nodules. Its specific activity increases during nodule development concomitant with the onset of biological nitrogen fixation (Reynolds et al., 1982). Following establishment of active nitrogen-fixing nodules, expression of the antisense constructs was induced by the addition of copper ions to the plant nutrient solution. This resulted in a dramatic decrease in AAT-P_2 enzyme activity and protein, with no compensating rise in AAT-P_1 enzyme activity. Concomitant with this decrease in enzyme activity, there was a decease of up to 90% in nodule asparagine concentration. These data establish a direct link *in vivo* between the proplastid-localised AAT-P_2 activity and asparagine synthesis in the nodule and suggest quite different roles in nodule metabolism for the two plant isoenzymes. The spatial and temporal control system demonstrated here will be of particular use in situations where antisense expression under control of constitutive or nodule developmental promoters would compromise nodule development itself and make physiological studies, such as that reported here, impossible.

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CHARACTERIZATION OF A 26KDa PROTEIN EXPRESSED IN SYMBIOTIC NODULES OF *Phaseolus vulgaris.*

NAYELI SANCHEZ GUEVARA, HECTOR E. PEREZ REYES AND FEDERICO SANCHEZ RODRIGUEZ.
Molecular Plant Biology Department. Biotechnology Institute, National University of Mexico. P.O. Box 510-3, ZP. 62271, Cuernavaca, Morelos, México.

Working on the characterization of actin from the *Phaseolus-Rhizobium* symbiotic system, we isolated a protein of 26kDa that has interesting biochemical properties. We decided to work on the characterization of this protein that co-elutes with actin in the purification procedure (Pérez, 1992) and here we present our preliminary data.

Our data show that this protein behaves as a high molecular weight product in native conditions (HPLC-TSK 3000), it is resistant to heat, acid soluble, with an acidic isoelectric point, and resistant to endogenous proteolytic degradation. All these characteristics resemble those of low molecular weight heat shock proteins (Vierlig, 1991).

In the nodule context, it is shown to be a nodule specific protein, that is expressed exclusively in the *Phaseolus-Rhizobium* system. The preliminary data showed that it is immunolocalized at the endomembrane level in infected cells, which is consistent to its capacity to adsorb to hydrophobic resins. Since this protein appears to expressed in late nodule development, it could be hypothesized that it may be expressed during the bacterial endosymbiotic process in plant host cells (Verma, et al., 1992) and possibly as a plant-defense response to them due to its stress protein features.

Our present aim is to know the sequence at the aminoacid and nucleotide level and compare them to other sequences in the literature in order to be able to propose a probable function.

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A GENE ENCODING A SUBTILISIN-LIKE PROTEASE IS EXPRESSED IN EARLY STAGES OF ACTINORHIZAL NODULE DEVELOPMENT

RIBEIRO, A.[1], AKKERMANS, A.D.L.[2], VAN KAMMEN, A.[1],
BISSELING,T.[1] AND PAWLOWSKI, K.[1]
[1]Dept. Molecular Biology, Agricultural Univ., Dreijenlaan 3, 6703
HA Wageningen,
[2] Dept. Microbiology, Agricultural Univ. Hesselink van Suchtelenweg 4,
6703 CT Wageningen, The Netherlands

To identify genes specifically expressed during early stages of actinorhizal nodule development, a cDNA library made from poly(A) RNA from root nodules of *Alnus glutinosa* was screened differentially with nodule and root cDNA, respectively. Seven nodule-enhanced and four nodule-specific cDNA clones were isolated. By using *in situ* hybridization, two of the nodule-specific cDNAs were shown to be expressed at highest levels in infected cells before the onset of nitrogen fixation; one of them, *ag12* (*A. glutinosa*), was examined in detail. Sequencing showed that *ag12* codes for a serine protease of the subtilisin (EC 3.4.21.14) family.

Subtilisins previously appeared to be limited to microorganisms (Siezen et al. 1991). However, recently , subtilisin-like serine proteases have been found in archaea, fungi, and yeast as well as in mammals (Barr, 1991), and even a plant subtilisin has been sequenced (Yamagata et al., 1994). In yeast and mammals, subtilases are responsible for the processing of peptide hormones (Barr, 1991). A homologue of *ag12*, *ara12*, was identified in *Arabidopsis*; it was expressed in all organs, at the highest levels during silique development. Hence, subtilases are involved in both symbiotic and non-symbiotic processes in plant development.

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SOYBASE 1995: NODULINS AND NITROGEN REDUCTION

SHOEMAKER, R.C.[1,2], AND IMSANDE, M.R.[1]
[1] Dept. of Agronomy, Iowa State Univ., Ames IA 50011, USA
[2] USDA-ARS-FCR, USA

SOYBASE, a soybean genome database, now contains approximately 100 megabytes of data. It employs a user-friendly, "point and click" hypertext system. SOYBASE recently was expanded to incorporate metabolic data. Forty-one pathways of nitrogen and fatty acid synthesis and degradation are diagrammed, revealing the respective enzymes, reactants, products, cofactors, kinetics, inhibition, activation, and regulation. More than 200 individual enzymes are detailed. Tags include EC number, gene symbol, purification, physical properties, source material, existence and accessibility of mutants, isozymes, sequence data, transgenic plants, and availability of DNA or antibody probes. Interconnections link pathways with enzymes. Furthermore, the entire database is interlinked through bold-faced, "clickable" hypertext items within any topic. Thirty-seven nodulins of soybean are summarized, showing related nodulins and sequences, degree of conservation, source, bacterium, time of initiation, possible function, physical characteristics, and availability of probes and transgenic plants. Additional material in the database includes 3 molecular RFLP maps, 1 classical map, 2-point genetic data, RFLP probes and images of autoradiograms, pathology data, storage proteins, and QTL studies. SOYBASE contains approximately 20,000 germplasm records with available trait data. Nitrate and nitrite reduction and their respective enzymes are currently part of SOYBASE, as are the synthesis and degradation of ureides. The database will be expanded to include biological nitrogen fixation, since this process distinguishes legumes from other crops. SOYBASE can be accessed 5 ways: 1) Remote login Telnet mendel.agron.iastate.edu (login as guest; password soybean); 2) Our World Wide Web connection through Mosaic (URL:http://mendel.agron.iastate.edu:8000/main.html); 3) Via WWW to National Agricultural Library through Mosaic or Netscape (URL:http://probe.nalusda.gov:8300); 4) By anonymous ftp from probe.nalusda.gov; and 5) Through our gopher server (gopher probe.nalusda.gov). Contact Marcia Imsande (mimsande@mendel.agron.iastate.edu) or Randy Shoemaker (rcsshoe@iastate.edu) with questions, comments, or contribution of data.

EXPRESSION OF URICASE GENE FAMILY IN SOYBEAN TISSUES & COMPARISON OF THE URICASES IN VARIOUS LEGUME NODULES

TAJIMA, S[1], TAKANE,K.[1], SEKI,K.[1], OHKAWA,K.[1], OKAZAKI,K.[1] and KOUCHI, H.[2]
[1] Department of Bioresourcescience, Faculty of Agriculture, Kagawa University, Kita-gun, Miki-cho, Kagawa 761-07, JAPAN, [2] Department of Applied Physiol., National Inst. Agrobiol. Resources, Tsukuba, Ibaraki 305, JAPAN

Uricase (EC 1.7.3.3), a major soluble protein in soybean nodules, is a key enzyme that irreversibly degrades purine base ring to produce allantoin. According the data of DNA cloning and immunoblotting analysis the uricase gene was expressed in soybean nodules and developing cotyledons. Genomic southern blotting data showed the uricase gene may be consisted of multi-gene family. For analyzing the mechanism of uricase gene transcription, we surveyed the genomic clone coding such uricase genes. We report here expression of the uricase genes and serological reactivity of uricases in various legume nodules (Taijima at al., 1992).

1. Positive clones were obtained from genomic DNA library of soybean plant using uricase cDNA clone as a probe. Sequencing analysis of the positive clones revealed that there are at least two kinds of clones (UR2 and UR9) encoding uricase gene. When the data was compared to that of (Nguyen et al. 1985) nucl. sequence of UR2 was similar to their data. When first exon regions of the two clones were sequenced, high homology was observed among the three clones in the sequences coding amino acids. In contrast, their 5'-upstream regions and the introns exhibited different sequences.
2. RT-PCR analysis using specific primer systems for UR2 or UR9 was employed to recognize each transcripts, showing both clones are expressing in nodules and in cotyledons. The conclusion was confirmed by restriction enzyme digestion and nucleotide sequencing of the RT-PCR products. Fifteen uricase cDNA clones isolated from soybean nodules were also analyzed by the same RT-PCR system. Eleven clones were of UR9 and four were of UR2.
3. Western blot assay using soybean uricase monoclonal and polyclonal antibodies showed the legume nodules of *Glycine max*, *Phaseolus angularis*, *Phaseolus vulgaris*, *Vigna unguiculata* L., *Vigna radiata* possessed uricase activity and serologically reactive to soybean uricase. *Canavalia gludiata* and *Lotus japonicus* uricase showed the weak reactivity. *Medicago sativa* and *Vicia sativa* nodules exhibited uricase activity, but no immuno-reactivity was observed. *Pisum sativum* ,*Vicia faba*, *Lupinus*, *Astragalus*,*Trifolium* nodules showed no uricase activity and no reaction to soybean nodule antibody. The data suggested that the uricase gene expression occurred in various legume plants that is not allantoin exporter.

Nguyen et al (1985) Proc Natl Acad Sci USA 82, 5040-5044.
Tajima, S. et al. (1992) New Horizons in Nitrogen Fixation, p 373

MODULATING THE EXPRESSION OF GLUTAMINE SYNTHETASE GENES IN A GENE MEMBER/ORGAN SPECIFIC MANNER: AN APPROACH TO UNDERSTAND THE REGULATION AND FUNCTION OF DIFFERENT GENE MEMBERS IN ALFALFA.

TEMPLE, S.J.[1], BAGGA, S.[1], KLYPINA, N.[1], Samac, D.[2] AND SENGUPTA-GOPALAN, C [1].
[1] Dept. of Agron. and Hort. New Mexico State University, Las Cruces, NM 8803, USA.
[2] Dept. Plant Pathology, University of Minnesota, St.Paul, MN, 55108, USA

Glutamine synthetase (GS) catalyses the first and key reaction in the assimilation of ammonia. It catalyses the ATP-dependent condensation of ammonia with glutamate to yield glutamine. Higher plant GS is an octameric enzyme encoded for by a small multigene family whose members exhibit organ/tissue specific patterns of expression. Two full length GS_1 cDNA clones representing distinct cytosolic isoforms of GS from alfalfa have been extensively characterized (pGS100 and pGS13). Significant differences observed in the expression profile between the two gene classes at an organ specific level probably represent differences both in the regulatory mechanism and functional role(s) of the two gene classes. We have introduced many gene constructs into transgenic alfalfa specifically aimed at both up and down regulating specific GS gene members or GS genes in an organ/tissue specific manner. Analysis of alfalfa transformants containing an antisense gene construct targeted to the 3' untranslated region of pGS100 showed a significant reduction in the level of related class endogenous transcript. Surprisingly these plants showed an overall increase in GS_1 polypeptide level and in total GS activity. Further analysis of these transformants revealed that two distinct gene compensation mechanisms may account for the increased activity. One involves the induction of a novel GS_1 gene which is normally silent, but is probably induced under conditions of high substrate availability. The second involves an increase in the level of translation or protein stability for the pGS13 subclass. No significant change in the level of pGS13 related transcript was observed. Vasculature specific down regulation of GS_1 produced few viable transformants. Survivors exhibited a characteristic yellowing in the growing tips. GS_1 transcripts (pGS100 and pGS13) showed up to 80% reduction suggesting that plants require only a low level of vasculature localized GS_1 transcript for viability.

HOST-INDUCED INEFFICIENCY IN *Pisum fulvum* IS OVERCOME BY A EUROPEAN *Rhizobium leguminosarum A-1.*

TCHETKOVA S. A. , KAMARDIN N. N., TIKHONOVICH I. A.
Research Institute for Agricultural Microbiology, Podbelsky Shosse 3, St. Petersburg-Pushkin 8, 189620, Russia.

Detailed screening of more than 250 pea cultivars of different geographical and botanical origin from the World Pea Collection of Vavilov Plant Industry Institute revealed only one type of ineffectivity attributed to the primitive *P. fulvum* Sibth. and Sm. from Israel. Four out of five cultivars formed small white nodules without acetylene-reducing activity when inoculated with a tester *R. leguminosarum* strain CIAM 1026 or when grown in unsterile soil at 25^0 C. Besides these plants demonstrated temperature sensitive nodulation forming practically no nodules at 18-20^0 C. Transmission electron microscopic analysis demonstrated typical traits of ineffectivity - unlike the effective variant bacteroids continued to proliferate, they were small, non-pleiomorphic and contained granules of PHB. In order to study genetic control of ineffectivity *P. fulvum* was crossed with 3 European *P. sativum* cultivars. Due to low compatibility (Errico et al., 1991) only 8 out of 35 *P. sativum* x *P. fulvum* crosses produced viable seeds. Reciprocal crosses with *P. fulvum* as a maternal partner were not achieved. F_1 hybrids when inoculated with CIAM 1026 at 20^0 C formed none or only 1-2 very big pink nodules thus pointing out to the presence of a dominant allele of temperature sensitivity and a recessive allele of ineffectivity. Back-crosses and F_2 hybrids are under study. Ineffectivity in *P. fulvum* is known to be overcome by F13 and other strains from Middle East, but never by a European strain (Lie, 1981). However *R. leguminosarum* A-1 isolated from St. Petersburg soils (Chetkova, Tikhonovich, 1986) formed large pink effective nodules on all *P. fulvum* cultivars both at 20^0 C and at 25^0 C thus overcoming not only ineffectivity but also temperature sensitivity. Strain A-1 is known to carry nodX (Ma, Iyer, 1990) and to overcome sym2-induced resistance in Afghan peas but unlike TOM (Winarno, Lie, 1979) is absolutely competitive and insensitive to nodulation blocking (Chetkova, Tikhonovich, 1986). Unlike F13 strain A-1 was effective on both *P. fulvum* and *P. sativum* plants.

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Lie TA (1981) Plant and Soil 61, 125-134.

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Pea seeds were provided by Dr. V. Serdyuk and F13 strain by Prof. T. A. Lie.
This work was financially supported by State Research Program INTERBIOAZOTE-2000, State Research Program Priority Directions of Genetics and Research Grant of International Science Foundation (Biodiversity).

DIFFERENTIATION AND REPRODUCTIVITY OF A BROAD HOST RANGE *BRADYRHIZOBIUM* SP. 97-1 IN NODULES OF PEANUT AND SOYBEAN

Junchu Zhou, Kongxin Liu, Runying Yang, Kaiman Peng and Xiaoqing Sun
Department of Microbiology, Huazhong Agricultural University, Wuhan 430070, P.R. China

The morphological differentiation and reproductivity of bacteroids isolated from nodules of two representative legume plants (peanut and soybean) infected by a broad host range rhizobium strain 97-1 were studied. Typical round shaped bacteroids were formed and their colony-forming ability (CFA) was not observed with the symbiosis between 97-1 and peanut. The CFA of small rods released from nodules was very low but increased with the age of nodules. Osmoprotection showed only a favorable effect on the CFA of small rods isolated from young nodules. Typical rod shaped bacteroids were also formed in soybean nodules infected by 97-1. The CFA of rods was also increased with the age of nodules but a detrimental effect was observed with the osmoprotection. The determinative effect of plants on the morphological differentiation and reproductivity of rhizobium bacteroids in nodules was directly proved by our results.

STUDIES ON PARA-NODULATION OF WHEAT AND NITROGEN FIXATION

CHEN W.-H., XIE Y.-X., CHEN T.-W.
Soils and Fertilizer Institute Chinese Academy of Agricultural Sciences
Beijing 100081 P.R.China.

Induction of *Azorhizobium* into the cells of non-legumes for symbiosis and nodulation.

In our experiments, nodule-like structures can be induced in wheat by low concentration of plant growth hormone, such as 2,4-D, chlorsulforn etc.. The induced nodules visible within 10-20 days after induction by 1 ppm 2,4-D solution and inoculated with *Azorhizobium*. In liquid culture 20-30 nodules were formed per plant, while in soil culture more than hundred nodules developed per plant. Ultrastructural observations by electron microscope have been shown that infected cells contained large number of rhizobia. The bacteria inside the cells of wheat nodules develop poly-β-hydroxybutyrate granules and occasionally, some peribacteroid membrane are found.

Nitrogen fixation of induced nodules of wheat by Azorhizobium.

We selected an oxygen-tolerant *Rhizobium Azorhizobium caulinodans* ORS571, as wheat inoculum. The maximum rate of acetylene reduction of infected wheat plants was approximately 100 nmol C_2H_4 per plant per day. In order to turther prove the nitroden fixation of wheat nodules we measured the origin of plant nitrogen by ^{15}N isotope dilution technique in pot experiment in 1990-1993.

Treated wheat grow normally and plant height, dry weight and N% are significantly higher than those of control plants.

Atom % ^{15}N excess in shoots of treated plants was lower than those of reference determined by ^{15}N isotope dilution method.

The studies on results of ^{15}N dilution method confirmed that about 8.85-22.69% of the plant nitrogen originated from the air.

NITROGEN FIXATION IN PARA-NODULES OF BARLEY AND MAIZE ROOTS BY INTRODUCED FREE-LIVING DIAZOTROPHS

KROL M.J., KOBUS J.
Department of Microbiology, Institute of Soil Science and Plant Cultivation, 24-100 Pulawy, Poland.

Introduction and methods

Para-nodules were induced on the roots of non-legume plant, using of 2,4-dichlorophenoxyacetate (2,4-D) and *Azospirillum* (Zeman et al. 1992; Christiansen-Weniger 1992; Christiansen-Weniger, Vanderleyden 1994). Barley and maize seeds were surface sterilized, using 0,2% $HgCl_2$ for 3 min. and inoculated with 0,1 mL of a 24-h culture (10^6 CFU) of *A.lipoferum* and *Azospirillum*-unlike* and different doses 2,4-D were added. The acetylene reduction assay (ARA) was used to test for the N_2-fixing capacity of the p-nodules, using 4-5 plants per vial (12 cm^3) containing 6 cm^3 of nitrogen-free Nfb (Baldani 1980). ARA was perfomed after 24-h incubation (atmosphere in the vials was 10% acetylene) on 14-days old seedlings. Para-nodules roots were incubated in the tetrazolium-phosphate buffer solution, containing 1,5g l^{-1} 1,3,5-triphenyltetrazolium (TPT) and 0,6g l^{-1} malate to localize the site of strong reduction. Such sites p-nodule were detached, crushed and examined by light microscopy.

Result and conclusions

The obtained results have proved that cereal roots with p-nodules formed in the presence of 1,0 and 1,5 mg 2,4-D kg^{-1} were about 5-times more active in the N_2-fixation, compared to the untreated roots. Nitrogen fixation (C_2H_2-reduction) was demonstrated in barley and maize root p-nodules induced by 2,4-D and inoculated with *A.lipoferum* amd *Azospirillum*-unlike. It was also shown that the host plant was albe to supply the necessary substrate required for the bacterial N_2-fixation (C_2H_2-reduction) within the p-nodules. Cytological investigations revealed that there were more azospirilla present within the p-nodules than in the root system. This indicates that the major part of ethylene production came from the azospirilla activity within the p-nodules.

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Christiansen-Weniger C (1992) Biol.Fertil. Soils. 13, 165-172.
Christiansen-Weniger C, Vanderleyden J (1994) Biol.Fertil.Soils 17, 1-8.
Zeman A et al. (1992) Res.Microbiol. 143, 847-855.

*- growth and nitrogen-fixing in presence of 3% and 5% NaCl.

PEA PHYTOGORMONE STATUS - A KEY POINT OF PLANT RESPONSE TO THE MICROBE TRANSFORMATION AND SYMBIOTIC FUNCTIONS

LUTOVA, L.A.(1) , SHAROVA, N.V. (1), KISLIN, E.N.(2),
PAVLOVA, Z.V.(1), CHERNYSH, N.S. (1)
1. Department of Genetics, St.Petersburg State University, 199034 St.Petersburg, Russia.
2. All Russia Research Institute for Plant Protection, St.Petesburg 189620, Russia.

The role of phytogormones in the symbiotic processes is well documented and widely discussed. Intraspecies variability for the symbiotic capacity among different legumes including pea (*Pisum sativum* L.) was described recently in our joint work with the ARRIAM Institute. The presented work was devoted to the elucidation of pea contrast forms differing in their morphogenic responses to wounding, exogenous phytogormones and oncotransformation. The genetical analysis of the genes controlling these morphogenic patterns was carried out.

Pea lines differing for symbiotic characteristics were used in this investigation. They were shown to vary in callus and root formation capacities on hormone free medium, but no shoot formation was observed. Phytogormones added decreased the level of heterogeneity in capacities for root and callus formation and made it possible to reveal genotype diversity for shoot formation, however, the level of shoot formation was very low. This was overcome by using modified method for regeneration from immature embryos which increased the level of shoot formation up to 100% in the case of shoot forming forms.

Interestingly, one of the pea forms studied demonstrated nontypical answer to oncotransformation - formation of roots on hormone free medium instead of tumorformation. The normal answer was restored by phytohormone treatment. It was shown that the capacities to transformation were inherited as a simple Mendelian traits: F1 hybrids were all root forming, while in F2 the segregation was revealed. Thus, the capacity to root formation is inherited as a dominant trait, while to callus formation - as a recessive one.

Using HPLC we have shown that genetic forms of pea, which displayed contrast morphogenic responses to oncotransformation, differed in the set of main endogenous cytokinins. We suggest that genotype differences governing the basic hormone level are responsible for the revealed heterogeneity in morphogenic capacities of pea forms.

The role of phytogormones in the determination of the plant responses to the different stresses including plant-microbe interaction may be studied successfully with pea lines differing for symbiotic characteristics.

SECTION 7

ENERGETICS OF NITROGEN-FIXING SYSTEMS. INTEGRATION OF CARBON, NITROGEN AND OXYGEN METABOLISM

INTEGRATION OF CARBON AND NITROGEN METABOLISM DURING PLANT-MICROBE SYMBIOSES: INTRODUCTORY REMARKS

COPELAND L.[1] AND ROMANOV V.[2]
[1]Department of Agricultural Chemistry and Soil Science, University of Sydney, NSW, Australia 2006
[2]A.N. Bach Institute of Biochemistry, Academy of Sciences, Leninsky pr. 33, Moscow, 117071, Russia

The reduction of N_2 to NH_3, inorganically or in biological processes, requires the input of a large amount of energy which is needed to break the very stable N≡N bond. The main source of reductant and energy for symbiotic nitrogen fixation in legumes is photosynthetically fixed carbon which is translocated into root nodules predominantly as sucrose. Numerous estimates have been made of the carbon cost of symbiotic nitrogen fixation, most of which are in the range between 5 and 18 g of carbon per g nitrogen fixed (eg Pate et al., 1981; Streeter, 1991; Vance and Heichel, 1991). In addition to providing energy and reductant for the nitrogenase reaction inside the bacteroids (equivalent to 16 ATP and 8 electrons per molecule of N_2 reduced), carbon is utilised in the assimilation of fixed nitrogen and for nodule growth and maintenance. On the basis of a requirement of 12 g of carbon per g of nitrogen fixed, Streeter (1991) has calculated that approximately 1.5 tonnes of sucrose/ha would be consumed to support the seasonal nitrogen fixation of a typical crop of soybeans. As much as 50% of the daily photosynthetic product may be consumed in the nodules during the peak nitrogen fixing period.

In return for supplying carbon to the microsymbiont, the host receives reduced nitrogen, mainly as NH_3, which effluxes from the bacteroids into the host cytosol to be assimilated, in the first instance into glutamine by the enzymes glutamine synthetase (GS) and glutamate synthase (GOGAT), and then into nitrogenous compounds for export (predominantly as amides in indeterminate nodules and as ureides in determinate nodules).

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The magnitude of these processess is quite remarkable, if one considers that they take place in a micro-aerobic environment where the concentration of free O_2 may range between 10 and 30 nM. The concentration of free O_2 in the nitrogen-fixing region of legume nodules must be strictly controlled, on the one hand so that it is readily available to the highly active metabolic processes that generate energy and reductant, but on the other hand so that it does not reach levels that cause damage to the O_2-sensitive nitrogenase enzyme complex within the bacteroids. Control of the free O_2 concentration is achieved by a combination of physical and biochemical mechanisms and is of critical importance to the effective functioning of nodules in symbiotic nitrogen fixation. The role of O_2 in root nodules is covered elsewhere in this volume.

Root nodules have a complex internal cellular organisation, and knowledge of the localisation of pathways of carbon and nitrogen metabolism is an important consideration to furthering our understanding of how these processes are integrated. The nitrogen-fixing region of nodules has large bacteroid-containing cells (ie infected cells) which are interspersed with smaller uninfected (ie bacteroid-free) interstitial cells. Infected and interstitial cells are in close contact, and have numerous plasmodesmatal connections for the symplastic transfer of metabolites (Brown et al., 1995). There is now a considerable amount of evidence, mainly from studies of symbioses that form determinate nodules, which suggests the pathways of carbon and nitrogen metabolism are compartmentalised. The initial stages in the utilization of sucrose and the final steps in the formation of ureides may take place to a greater extent in uninfected cells, whereas early steps in the assimilation of fixed nitrogen occur mainly in infected cells (reviewed Schubert, 1986; Day and Copeland, 1991; Gordon, 1992).

Bacteroids in infected cells are surrounded by the peribacteroid membrane which isolates them from direct contact with the cytoplasm of the host. Organizational units formed by the enclosure of bacteroids in the peribacteroid membrane are known as symbiosomes, which may contain only a single bacteroid or up to 20 bacteroids depending on the type of symbiosis. The peribacteroid membrane has an important role in regulating the transfer of metabolites between the host and microsymbiont: all solutes that are transferred between the two partners must cross the peribacteroid membrane (as well as the bacteroid membrane), which may involve specific translocator proteins and the input of energy.

In an effective symbiosis, the delivery of photosynthate to the nitrogen-fixing region in nodules, and its subsequent conversion in the host fraction to substrates for the bacteroids and carbon skeletons for assimilation of fixed nitrogen, needs to be coordinated with the demand for energy and reductant for nitrogenase within the bacteroids. These processes are linked by the exchange of metabolites between the host and microsymbiont across the peribacteroid membrane. To gain a better understanding of how carbon and nitrogen metabolism are integrated during nitrogen fixation, much more needs to be learnt of control mechanisms at the genetic and enzymic levels that regulate the metabolic processes concerned in the host and bacteroid compartments and at the peribacteroid membrane interface between the two symbiotic partners.

The papers in this section describe recent progress towards characterising some of these control mechanisms. The presentations will include: an overview of metabolic pathways of carbon and nitrogen metabolism in nodules and hypotheses as to why the microsymbiont fixes nitrogen and releases it to the host legume (M. Kahn); sucrose metabolism to support symbiotic nitrogen fixation, and possible affects on nitrogen fixation of alterations in the activity and level of gene expression for sucrose synthase (A Gordon); the role of malic enzyme(s) in the utilisation of dicarboxylic acids by bacteroids (T Finan); functional analysis and regulation of expression of glutamine synthetase genes in legumes (C Sengupta-Gopalan); and the role of the peribacteroid membrane in interactions between the symbiotic partners, covering pH regulation in the peribacteoid space (N Kudryavtseva), the nature of metabolites exchanged across the peribacteroid membrane (D Day), and characteristics of a proton-pumping ATPase in the peribacteroid membrane (H Haaker)

Finally, it is worth noting that a better understanding of how carbon and nitrogen metabolism are integrated during symbiotic nitrogen fixation will have relevance to other areas of plant biology. The utilization of sucrose to provide respiratory substrates and the assimilation of inorganic nitrogen are ubiquitous metabolic processes. Although the pathways concerned have been described in textbooks for many years, there is still only limited knowledge of regulatory mechanisms, of how fluxes through different pathways are coordinated, of metabolite translocation across membranes to link pathways in different compartments, and of signal-transduction mechanisms that bring about changes in metabolism in response to external influences. No doubt, answers to some of these questions will come from genetic and enzymic studies of symbiotic nitrogen fixation in legumes, a system in which a host and microsymbiont interact to effect an energetically-demanding and chemically-difficult reaction.

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CARBON METABOLISM IN THE RHIZOBIUM- LEGUME SYMBIOSIS

KAHN M.L., MORTIMER M., PARK K.S., ZHANG W.
Institute of Biological Chemistry, Departments of Microbiology and Genetics and Cell Biology, Washington State University, Pullman, Washington 99164-6340.

Combined nitrogen is often the nutrient that limits plant growth. Legumes that establish a nitrogen-fixing symbiosis with rhizobia can gain a major competitive advantage, since they can then grow well on nitrogen-limited soils and can allocate more nitrogen to their seeds and tissues in the form of protein and nitrogen-containing feeding deterrents. The high protein content of legumes makes them important grain and forage crops that may become even more valuable through manipulation of the symbiosis. By allowing legumes to prosper without expensive and energy requiring inputs of nitrogen fertilizer, the symbiosis is also crucial to many ideas for sustainable agriculture.

Symbiotic nitrogen fixation takes place in nodules, specialized organs that develop after rhizobia infect a growing root. Within the infected cells of the nodule, differentiated forms of rhizobia, the bacteroids, fix nitrogen and produce enough ammonia to supply the growing plant. Although free-living bacteria fix nitrogen only when they are growing under nitrogen-limited conditions, symbiotic rhizobia continue to fix nitorgen even after they have produced enough ammonia to satisfy their own nitrogen requirements. We have argued (Kahn et al. 1985; Udvardi, Kahn 1992) that rhizobia are unlikely to be altruistic and therefore must have some motivation other than obtaining ammonia to continue carrying out the energy intensive nitrogen fixation reactions. In this view,symbiotic nitrogen fixation is not coupled to a bacterial need for ammonia but instead it relieves some other stress that limits bacterial growth. By relieving this other stress, rhizobia directly benefit from nitrogen fixation. Several models have been proposed to explain how this interaction could work (Kahn et al. 1985; Kohl et al. 1988; McDermott et al. 1989; Mellor 1989; Brewin et al. 1990; Udvardi, Kahn 1992; Hunt, Layzell 1993) but all depend on the idea that nitrogen fixation is maintained in a biochemically defined and predictable environment, where the plant has considerable control over the nutrients available to the bacteria and the removal of catabolic byproducts. In this discusssion, we will focus on models of symbiotic metabolism in which carbon availability, oxygen concentration or pH play a major role as stresses that could influence nitrogen fixation. However, although it has been convenient to consider these stresses independently, they are obviously linked through metabolism.

The outline of our understanding of the general flow of mebolism in the nodule has been described for some time (Atkins 1991; Streeter 1991; Vance, Heichel 1991).

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Photosynthetically derived carbon compounds, such as sucrose, enter the nodule from the phloem. In the plant compartments of the nodules there are converted to compounds, such as organic acids and amino acids, that are transported through the peribacteroid membrane to the bacteroids. Futher catabolism by the bacteroids generates ATP and reductant used by nitrogenase to convert dinitrogen into ammonia. The ammonia produced is released to the plant where it is assimilated by glutamine synthetase and ultimately converted into amides or ureides for export to the rest of the plant (Atkins 1991). However, to understand nodule metabolism we need a more complete understanding of the details of these processes and of the interactions between them.

The nitrogenase reaction (Appleby 1984) is at the center of the picture.

$$N_2 + 16ATP + 8e^- + 10H^+ \longrightarrow 2NH_4^+ + H_2 + 16ADP + 16Pi \quad (1)$$

Carrying out this reaction is very demanding, not only because it requires a large amount of ATP and low potential reductant, but also because it is sensitive to inactivation by oxygen and oxidants. Rhizobia are aerobes and a major fraction of the ATP produced in support of nitrogen fixation is probably produced by oxidative phosphorylation. It has been shown in *Bradyrhizobium japonicum* that a microaerobically induced cytochrome complex is needed for symbiotic competence (Preisig et al. 1993). Rhizobial respiration is the major oxygen sink in the nodule and low K_m values have been reported for the bacteroid terminal oxidases (Hunt, Layzell 1993). This oxygen sink, together with a variable diffusion barrier located in the nodule parenchyma, lowers the oxygen concentration to a level compatible with nitrogenase activity (Hunt, Layzell 1993). Leghemoglobin, an oxygen binding protein found in millimolar concentrations in the plant cytoplasm, increases the diffusion of oxygen through the cytoplasm to the bacteroids by increasing its effective solubility (Appleby 1984). Leghemoglobin also serves as a buffer to moderate changes in oxygen concentration resulting from changes in the permeability of the diffusion barrier or the respiration rate.

The low oxygen concentration in nodules limits metabolism. In experoments in which the external oxygen concentration is increased moderately and quickly, bacterial respiration and nitrogen fixation also increase (Hunt, Layzell 1993). Thus, models in which the bacteria fix nitrogen in order to obtain more oxygen and achieve a higher catabolic efficiency are attractive. In these models, the "boundary condition" where the diffusion barrier is high and leads to extreme oxygen deprivation is likely to be stable because, in this state, nitrogenase would not be inactivated and would be able to resume nitrogen fixation as nirmal permeability is restored. In contrast, models in which carbon available to the bacteroid is limited suffer from the problem that, if the bacteria run out of oxidizable compounds, they cease to be a sink for oxygen. The oxygen concentration in nodules would then rise and the oxygen lability of nitrogenase would become important. Carbon compounds stored in the bacteroids, such as poly-β-hydroxybutyrate, could be used as a buffer to prevent this from happening (Bergersen, Turner 1990).

Photosynthate enters the nodule as sucrose but genetic and biochemical evidence shows that bacterial catabolism of mono or disaccarides is not essential for the bacteria to be effective (McDermott et al. 1989; Streeter 1991; Vance, Heichel 1991). Instead, sugars are converted to organic acids before being made available to the bacteroids, probably by using pathways more often associated with fermentation than oxidation (Vance, Heichel 1991). It has argued that the terminal oxidase in the plant mitochondrial electron transport chain, which has a K_m for oxygen of about 100 nM, cannot effectively participate in oxidative metabolism at the oxygen concentrations found in nodules, forcing the use of alternate electron acceptors. Evidence suggests that fermentation might follow a scheme in which phosphoenolpyruvate (PEP) is converted by PEP carboxylase to oxaloacetic acid (OAA) and NAD is regenerated by the reduction of OAA to malate or aspartate. The increase in plant PEP carboxylase and the rapid incorporation of $^{14}CO_2$ into malate and aspartate shows that this pathway is induced and active in nodules (Rosendahl et al. 1990; Vance, Heichel 1991).

There are two major fates for the dicarboxylic acids. Some are fed to bacteroids and are oxidized using electron transport chains that terminate in the bacteroid's high-affinity oxidases. This catabolism would ultimately provide the reductant and energy for nitrogen fixation. Consistent with this view, it has been shown that the soybean peribacteroid membrane can transport dicarboxylic acids and that bacterial mutants with defects in dicarboxylic acid transport are ineffective (Ronson, Primrose 1979). Other dicarboxylic acids are used to make nitorgen-containing compounds, such as asparagine and glutamine, that are exported from the nodule. Amino acid synthesis can also promote organic acid catabolism by consuming NADH and removing organic acids, such as pyruvate, OAA and oxoglutarate, that accumulate and inhibit further metabolism.

Bacterial TCA cycle metabolism. Although it is thought that the bacterial TCA cycle is necessary to generate energy for symbiotic nitrogen fixation, the genetic evidence implicating the decarboxylating leg of the pathway is weak and the biochemical evidence is ambiguous. Isocitrate dehydrogenase (IDH) and oxoglutarate dehydrogenase (OGDH), the two decarboxylating enzymes in the TCA cycle, are often sensitive to inhibition by NAD(P)H and their expression and activity are inhibited by anaerobic conditions. McDermott et al. (1989) speculated that reductant accumulating in the microaerobic nodule environment would inhibit IDH and OGDH and that low enzyme activity would divert the flow of carbon to poly-β-hydroxyburate, the dicarboxylic acid pathway or glutamate, depending on whether citrate synthase (CS), IDH or OGDH was limiting.

We have cloned genes that encode enzymes of the decarboxylating leg of the *Rhizobium meliloti* TCA cycle by complementing various *E.coli* mutants with corresponding defects. These include the genes that encode citrate synthase (*gltA*), isocitrate dehydrogenase (*icd*) and the sucA and sucB components of oxoglutarate dehydrogenase. Isolation and characterization of the *icd* gene and the mutants derived from it has been published (McDermott, Kahn 1992).

The *R.meliloti* citrate synthase gene (*gltA*) was cloned based on its ability to complement an *E.coli* mutant that lacked citrate synthase and was a glutamate auxotroph. Preliminary DNA sequence analysis shows that the *R.meliloti* 104A14 *gltA* amino acid sequence is 63% identical to the *E.coli gltA* sequence and 84% identical to the *R.tropici gltA* sequence (Pardo et al. 1994). A *gltA* mutant was constructed by inserting a kanamycin resistance gene into *gltA* then recombining this allele into the bacterial chromosome. This mutant is a glutamate auxotroph and does not revert or pseudorevert to Glt^+, suggesting that *R.meliloti*, unlike *Rhizobium tropici* (Pardo et al. 1994), has only one copy of the *gltA* gene. The mutant grows well on oxoglutarate, glutamate or arabinose, which is metabolized to oxoglutarate. When various carbon sources were added to media that contained enough arabinose to satisfy the auxotrophy but not enough to support growth, the mutant was able to grow as well as wild type on many sugars and organic acid but not on pyruvate. Surprisingly, it grew poorly on glutamate and mannitol, and very poorly on oxoglutarate and mannitol, suggesting that transport of glutamate and oxoglutarate are under some kind of catabolic repression.

Table 1. Symbiotic phenotypes of *R.meliloti* citrate synthase mutants.

Strain	Phenotype bacteria (plasmid)	Nodules/ plant	Nodule color	Weight/ plant (mg)
104A14 (pCPP30)	Wild type	6.4+2.7	pink	19
CSDX1 (pCPP30)	CS-	4.3+2.1	white	4
CSDX1 (pMK426)	CS-(CS+)	7.9+2.0	pink	16
104A14 (pMK426)	Wild type (CS+)	7.9+2.0	pink	21
no inoculum	0	4		

CSDX1 is an insertion/deletion mutant of 104A14. pMK426 is a derivative of the vector plasmid pCPP30 that carries the *R.meliloti* citrate synthse gene.

The *gltA* mutant forms a normal number of nodules on the roots of infected plants but these are white and don't appear to contribute to plant nutrition (Table 1). Insertion of a plasmid borne copy of the *gltA* gene into the *gltA* mutant complements the symbiotic defect. However, despite a 10-fold increase in citrate syntase specific activity in *gltA* or wild-type cells containing the plasmid, the number of nodules formed on plants is about the same as formed by the wild type. This contrasts with the situation in *R.tropici*, where one of citrate synthase is induced under symbiotic conditions and is neede for the formation of normal numbers of nodules.

The *gltA* mutant's LPS appears to be altered - colonies on calcofluor medium (Leigh, Walker 1994) are bright while rather than yellowish and the fluoresscence of the colonies under UV light is blue-white instead of the yellowish-green of the wild-type. This phenotype is not seen with an *icd*::Tn5 mutant but is seen in the *icd gltA* mutant described previously. It therefore appears that the citrate synthase defect is itself responsible for the blue-white florescence, rather than this phenotype being a general defect in mutants in the decarboxylating leg of the TCA cycle. Because mutants with altered LPS often form defective nodules, it may not be possible to assign the *gltA* mutant's defect in nitrogen fixation to altered energy generation by the TCA cycle.

Plant carbon metabolism. Plant enzymes are involved in carbon metabolism that supports symbiotic nitrogen fixation. We have focused on NADP-dependent isocitrate dehydrogenase which propaply synthesizes oxoglutarate that is used in the synthesis of the glutamate family of amino acids. We have described a cDNA clone, pTM4, which encodes an NADP-IDH from soybean that is predicted to have about 69% identity with an NADP-IDH from pig (Udvardi et al. 1993). Three additional cDNA clones have been isolated that appear to be from separate genes. The DNA sequence of the NADP-IDH from a second full-length clone, pKS1, predicts a protein with about 80% identity to the pTM4 protein. Both of these sequences have unusually long leader sequences that suggest that they may encode organelle forms of NADP-IDH.

Preliminary results suggest that a third clone, pKS2, encodes a protein that is more closely related to the pKS1 protein than to the pTM4 protein.

Both the pTM4 and pKS1 proteins have been overproduced in *E.coli* as His_6 tagged proteins and purified to homogeneity by affinity chromatography. Characterazation of the enzymes is in progress and an attempt is being made to crystallize one of them. Antibodies to both proteins have been raised in rabbits and these are quite specific for the original antigen-the antibody to pTM4 protein does not react with the pKS1 protein and the pKS1 antibody reacts only very weakly with the pTM4 protein. Because the two proteins are predicted to be so similar, we had feared that there would be considerable cross-reactivity between the anti- bodies but the low level of cross reactivity suggested that the highly conserved regions may be seen by the rabbit immune system as self-antigens and so limit the antibodies produced to the less conserved regions. The pTM4 antibody reacts with a protein of the expected molecular weight in root, stem, nodule and cotyledon tissue; pKS1 antibody reacts with all of these tissues except root, showing the best evidence to date of tissue-specific expression of IDH. Neither antibody is very active against leaf proteins, suggesting that the leaf proteins are encoded by other genes.

Generation of reductant. The nitrogenase reaction requires a low potentional reductant. It seems likely that a reduced ferredoxin or flavodoxin (Fd_{red}) is the ultimate carrier of reductant to nitrogenase. But how is Fd_{red} produced?

In *Klebsiella pneumoniae*, the best defined nitrogenase system, reduced flavodoxin is produced through the reversible decarboxylation of pyruvate using the enzyme pyruvate-flavodoxin oxidoreductase. Similar substrate-level generation of reductant has been implicated in nitrogen fixation in a number of other systems. The oxidation of aldehydes or oxoacids, such as pyruvate and oxoglutarate, has sufficient potential to drive the reduction of a low potential electron carrier. It has also been speculated that ferredoxin could be using NADH or NADPH as an electron donor, even though the reduced/oxidized ratio of the pyridine cofactors is not high enough to drive this reaction very far. The mechanism of generating reductant has consequences for other aspects of rhizobial metabolism. If NADH or NADPH can be used, then many cellular reactions could be used to contribute to nitrogen fixation. On the other hand, if only substrate level reactions can generate reductant, then relatively few reactions, such as pyruvate-ferredoxin oxidoreductase, would be involved. Because of the number of electrons that would need to be delivered to nitrogenase, cellular metabolism might be organized to generate these substrates. The requirement of an NAD dependent malic enzyme for fixation in *R.meliloti* (Drisscoll, Finan 1993) may be evidence for such a pathway. The other consequence of specialized, substrate level generation of reduced ferredoxin is the need to deal with the carbon-containing products of the reaction. If, for example, pyruvate-ferredoxin oxidoreductase was used to generate reduced ferredoxin, a major sink for acetyl CoA would also be needed. As pointed out above, the microaerobic environment may limit the extent to which the TCA cycle can serve this function and it would be expected that acetyl CoA would be converted to poly- β -hydroxybutyrate (Bergersen, Turner 1990) or that it could be used to make ATP using acetyl phosphate as an intermediate.

Hydrogen ions. In considering the nitrogenase reaction, we often ignore the consumption of hydrogen ions that accompanies the generation of ammonia. However, free-living forms of rhizobia are often very sensitive to low pH (Perez-Galdona, Kahn 1994) and even moderately acid conditions can limit bacterial growth and the initiation of nitrogen-fixing nodules on the roots of host plants. Within the plant cell, symbiotic bacteria or bacteroids may also have to cope with relatively acid conditions. Bacteroids are enclosed within a plant-derived symbiosomal membrane and it has been found that symbiosomes contain a number of acid activated hydrolases (Mellor 1989). It has been argued that the symbiosome is like a vacuole and vacuoles are thought to have internal pH values between 5.5 and 6.0. Transport of protons or the protonated forms of organic acids into the symbiosome would acidify the compartment (Udvardi, Kahn 1992). Brewin et al. (1990) have proposed that bacteroids may counteract this acidification of the peribacteroid space by synthesizing NH_3 or by catabolizing dicarboxylic acids, which, in free-living *R.meliloti* leads to alkalization of the medium. The nitrogenase reaction consumes at least 8 H^+ per N_2 fixed. Thus, nitrogen fixation itself may be a substantial sink for protons pumped into the peribacteroid space by the plant. If acetate is produced, it could also be used to remove protons since, as a permeant ion, it would move relatively easily across the membranes and its final distribution would depend on whether the plant was acting as a sink for acetate. We have shown that *R.meliloti* is sensitive to relatively low concentrations of monocarboxylic acids and moderate pH, which are bacteriostatic (Perez-Galdona, Kahn 1994). Whatever the mechanism for removing protons from the symbiosime, it is clear that a dynamic equilibrium must exist in order to hold the pH of this compartment at a level suitable for bacteroids to function.

Ditta et al. (1987) observed that the *nifH* promoter was induced by pH<6.0. We have investigated the transduction pathway for this effect and determined that it is affected by acetate, implying either that acetate interacts with an external sensor or that low internal pH is leading to induction. Late logarithmic phase cells of *R.meliloti* containing various promoter-*lacZ* fusions were grown in ammonia and glutamine rich media. They were then washed and resuspended in buffered malate medium lacking a nitrogen source but containing X-gal, mixed with agar and poured into glass vials. The development of color was followed with time. In the presence of 10 mM acetate, a band of color is observed close to the surface at pH 6.0 but deeper in the agar as the pH is lowered. Color development begins within one hour and we do not think that differential cell growth is responsible for the band formation. The bands formed by the *nifA-lacZ* and *nifH-lacZ* fusions are very similar. The band formed in an *ntrC* background is more diffuse but as intense as in *ntrC*$^+$. A much less intense band is formed in a *nifA* mutant background. Several factors suggest that oxygen is a major variable in determining band intensity and distribution. Direct measurement of the oxygen concentration in the agar using a syringe needle style microelectrode showed that an oxygen gradient was established within the first hour and was stable for at least 16 hours. The band position was typically at about 30-50% of the concentration of air saturated buffer, considerably higher than the microaerobic conditions often used to induce *nifH-lacZ* fusions. The formation of a band suggests that there is a relatively narrow window of appropriate conditions for inducing the fusions.

Integrating the carbon, oxygen and pH models. Although carbon, oxygen and pH have been described as separate influences but they are clearly linked through metabolism. Limited oxygen availability can lead to greater used of fermentation pathways and affect pH. In animals, lowered pH decreases oxygen binding by hemoglobin and leads to the release of oxygen in tissues where excess CO_2 and lactin acid are accumulating. Although purified leghemoglobin does not have a Bohr effect, nicotinic acid binding to leghemoglobin displaces oxygen from leghemoglobin-oxygen complexes more easily as pH decreases (Appleby et al. 1973). The effect is strong and remarkably similar in various leghemoglobins (Atanosov et al. 1989), causing the pK for nicotinate to increase between 0.6 and 1.1 for every unit decrease in pH (pH 5-7). Although the nicotinate concentration in soybean nodules is lower than previously though (Klucas, Appleby 1991), it is comparable to the solubility of oxygen in water and localized or kinetic effects could contribute to a modified Bohr effect. Acetate (Krasnobaeva, Atanosov 1979) can also bind leghemoglobin. Even weak binding of metabolites could be important because of the tremendous difference between the concentrations of oxygen bound to leghemoglobin and free in the cytosol. It is possible to imagine that pH driven changes in oxygen availability could be part of a homeostatic mechanism that is involved in regulating the fraction of carbon that is oxidized or fermented. As oxygen concentrations decrease, acids released by the incomplete oxidation of sucrose would lower the pH, releasing more oxygen from leghemoglobin and leading to more respiration. This immediate feedback could be important in maintaining a stable rate of metabolism.

There is also the possibility that the interaction could be more subtle. Consider two fermentation/oxydation schemes for the conversion of malate to acetate (Fig.1). Both produce acetate and release it as a neutral fermentation product into the plant cytoplasm where we speculate it dissociates and the lower pH causes oxygen to be released. In the first pathway, malic enzyme and pyruvate dehydrogenase are used and two NADH molecules are produced. The second pathway uses malic enzyme and pyruvate-ferredoxin oxidoreductase and only one NADH is produced, along with one Fd_{red}.

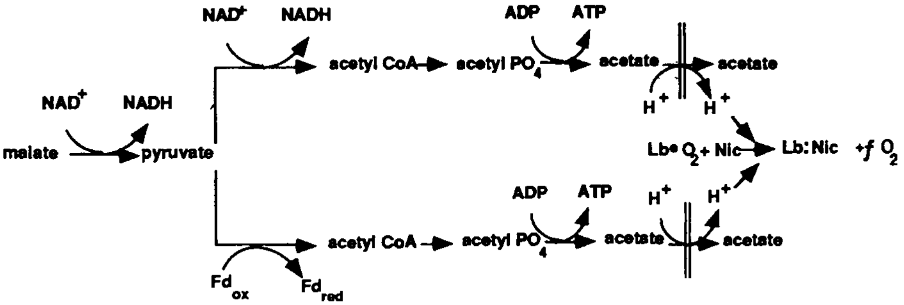

Figure 1. Two alternate pathways of malate degradation. The fraction of oxygen released by leghemoglobin (*f*) will depend on buffers in the cytosol, the concentration of nicotinate and the magnitude of the "Bohr" effect on the leghemoglobin-oxygen complex.

What if less than one oxygen molecule is released per acetate, as a result of buffering in the cytosol or the size of the Bohr effect? Then the first pathway will not be able to regenerate all of the NAD^+ and will be unable to repeat the process. However, the second pathway can return to the original oxidation state of the cofactors if the reduced ferredoxin is used to fix dinitrogen. Fixation costs 2 ATP per electron transferred to nitrogen but if two or more ATP can be made from NADH oxidation, using the second pathway will be profitable since 1 ATP comes from acetyl phosphate. If 3 ATP are made, there is sufficient profit without the need for acetate excretion.

The second pathway is an example of a more general speculation, that under some set of conditions the use of nitrogen as a terminal electron acceptor might actually be advantageous, despite the high ATP cost of nitorgen fixation. We have been unable to contrive a pathway that accomplishes this goal without the co-metabolism of oxygen- the yield of ATP from fermentation is too low. But if the pathway for carbon catabolism requires more than one oxidation and if one of these oxidations produces a low potential electron that can be used by nitrogenase, the profit from being able to generate more ATP from electron transport to oxygen than is consumed during electron transport to nitrogen could be significant, particularly if leghemoglobin plays a direct and active role in couling metabolic pathways to oxygen availability.

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SUCROSE METABOLISM TO SUPPORT N_2 FIXATION IN LEGUME ROOT NODULES.

GORDON A.J.
Institute of Grassland & Environmental Research, Plas Gogerddan,
Aberystwyth, Dyfed SY23 3EB. UK.

Introduction

Legume nodule N_2 fixation is entirely dependent on the direct or indirect supply of sucrose from photosynthesis. Sucrose is delivered into the cortex of the nodule via the phloem from which it must be unloaded and then diffuse to the metabolically active cells involved with provision of carbon for energy production in the N_2 fixation process and for the synthesis of N products (Gordon, 1992). This paper will focus on the enzymes of sucrose metabolism in nodules, their location and involvement in vivo, the ways in which they may be regulated and finally their potential role in modulating N_2 fixation and in sensing whole plant stress status.

Sucrose metabolising enzymes in nodules

Mature legume nodules contain both alkaline invertase (AI*) and sucrose synthase (SS) with no, or very little acid invertase (Robertson, Taylor, 1973; Morell, Copeland, 1984, 1985; Gordon, 1992). Sucrose synthase catalyses a freely reversible reaction (see Fig. 1) which in the degradative direction produces UDPG and fructose from the substrates sucrose, UDP and H^+. AI catalyses an irreversible reaction to produce free hexoses. The products of both reactions can enter glycolysis, UDPG via UDPGP and PGM, and fructose following phosphorylation by FK. The latter enzyme may use UTP and so recycle UDP for the SS reaction. Phosphorylation of the hexoses (from AI) would produce hexose 6-phosphates which are readily interconverted by PGI. Generally, HK activity in nodules is considerably lower than that of FK (Copeland, Morell, 1985).

***Abbreviations**: AI, alkaline invertase; AAT, aspartate amino transferase; FK, fructokinase; F6P, fructose-6-phosphate; $F16P_2$, fructose 1,6 bisphosphate; FS, fluorosucrose; G1P, glucose-1-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; GS, glutamine synthetase; HK, hexokinase; Lb, leghaemoglobin; MDH, malate dehydrogenase; PEPC, phosphoenol pyruvate carboxylase; PFK, phosphofructokinase; PGM phosphoglucomutase, PGI, phosphoglucose isomerase; pO_2 partial pressure of O_2; PPi, inorganic pyrophosphate; SS, sucrose synthase; UDP, uridine diphosphate; UDPG, UDP glucose, UDPGP, UDPG pyrophosphorylase; UTP, uridine tri-phosphate.

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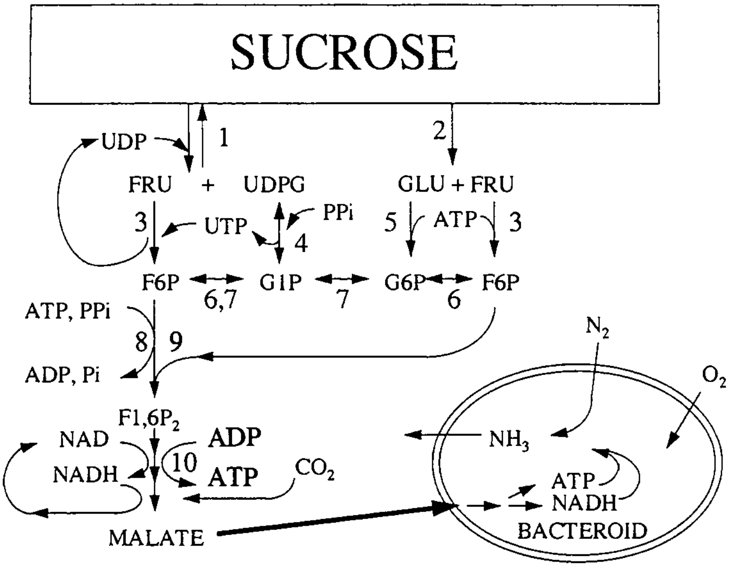

Fig. 1. Sucrose metabolism to supply carbon to bacteroids.
Enzymic steps: 1, SS; 2, AI; 3, FK; 4, UDPGP; 5, HK; 6, PGI; 7, PGM, 8 & 9 ATP- and PPi-PFK; 10, glycolytic enzymes plus PEPC and MDH.

The step from F6P → $F16P_2$ can be catalysed by a further pair of similar function enzymes:- ATP- or PPi-dependent PFK. The relative involvement of the two enzymes is not known. However, if ATP is in short supply (restricted mitochondrial function due to low pO_2?) and PPi abundant then the SS route (involving the two PPi-dependent enzymes UDPGP and PPi-PFK) is much more energetically efficient than the AI, HK, ATP-PFK route. Our measurements of PPi content in nodules suggest that the concentration may be c.300 μM in the cytoplasm (Gordon, James - unpublished). Metabolism of $F1,6P_2$ is likely to involve the normal glycolytic enzymes (all of which are present - Copeland et al, 1989; Kouchi et al, 1988) plus PEPC and MDH. The latter step ensures that NADH generated in the glyceraldehyde-3-phosphate dehydrogenase reaction is re-oxidised to NAD. The role of malate and other substrates for bacteroid metabolism will be addressed in other papers in this symposium. It seems very unlikely that bacteroids can use sucrose directly (Streeter, 1991, Day et al 1989).

Location of sucrose metabolism

SS and AI are cytosolic enzymes which occur predominantly in the infected region of mature soybean nodules (Gordon, 1991). Both enzymes occur in both infected and uninfected cells (Kouchi et al, 1988) although recent immunological data suggest that SS may be more concentrated in uninfected interstitial cells of the infected region, and in the cortical cells abutting infected cells, than in infected cells themselves (Gordon et al, 1992, 1995; Zammit, Copeland, 1993). Nonetheless, because infected cells are far more numerous than uninfected interstitial cells and contain more cytoplasmic volume and protein (Bergersen 1982, Gordon, 1991) it is likely that infected cells contain the bulk of the SS activity found in soybean nodules. We have argued, nonetheless, that because of the greater concentration of SS in cortical cells in close proximity to vascular bundles, and in uninfected interstitial cells of the central region that these cells are probably the main route of sucrose movement into the active central region (Gordon et al, 1992, 1995). Moreover, this is especially likely considering the relative numbers of plasmodesmata connecting the various cell types in the nodule (Selker, 1988) which were greatest between adjoining uninfected cells, fewer between infected: uninfected cells, and much fewer between adjoining infected cells.

Involvement of SS and AI in sucrose metabolism in nodules

An intriguing question in plants is why there are different enzymes which perform essentially the same task. In the case of sucrose metabolism in legume nodules SS or AI could be involved as outlined above. In active soybean nodules the flux of sucrose to account for the rate of N_2 fixation, nodule respiration and N-product export is approximately 0.4 μmoles sucrose g^{-1} nodule fresh weight min^{-1} at 30 °C, while the maximum catalytic activities of both SS and AI in soybean nodules are c.4-6 μmoles g^{-1} fresh weight min^{-1} at 30 °C, i.e. ten fold greater than the estimated rate of utilisation (Gordon, 1991). However, rates in vivo are likely to be constrained by the concentrations of substrates, products and other modulators, which at this stage we can only speculate about (see later).

To assess the contribution that SS and AI make to in vivo sucrose metabolism we compared the metabolism of [^{14}C-glucosyl]-sucrose and [^{14}C-glucosyl]-1-fluoro-1-deoxy fructosyl sucrose (FS) in detached soybean nodules in closed cuvettes under an argon/oxygen (80:20) gas mixture. The rationale behind the experiment is that FS is metabolised to different extents by SS and invertase when compared with sucrose (Hitz et al, 1985; Schmalstig, Hitz, 1987). The results suggest that in nodules of young plants (3-7 weeks after sowing) SS was almost entirely responsible for sucrose metabolism. In older nodules SS was involved to a lesser extent (responsible for 60% and 40% of sucrose metabolism at 11 and 14 weeks, respectively). These data do not correspond with the measured activities of SS and AI since the calculated relative activities (%SS: %AI) at pH 7.5 were 57:43, 54:46, 45:55 and 33:67 at 3,7,11 and 14 weeks and imply that SS is involved in sucrose metabolism to a much greater extent than would be predicted from observations of maximum catalytic activity.

Properties of the enzymes in vitro

What are likely to be the principal means of controlling sucrose utilization by SS and AI in vivo? Fine control could be exerted by changes in concentrations of effectors in the cytoplasmic environment.

pH

H^+ is a substrate in the hydrolysis of sucrose by SS; thus cleavage is favoured at low pH while synthesis of sucrose is more likely at high pH. This is reflected in the pH optima for the cleavage and synthesis directions of 6.8 and 8.5, respectively (Harrison, Gordon, in press; Morell, Copeland, 1984, 1985). In addition, AI has optimum activity at c.pH 7.8. Thus at high pH a futile cycle could exist whereby sucrose, synthesised by SS, could be hydrolysed by AI. The net outcome could be wasteful use of ATP and production of PPi and H^+.

Shortage of NTP could result in hexose accumulation and inhibition of AI and SS (see below).

Low pH would favour the cleavage of sucrose by SS and diminish its role in sucrose synthesis and, in addition, reduce the contribution of AI to sucrose hydrolysis. Whether significant changes occur in the pH of the nodule cell cytosol as a result of stresses perceived by the plant, or during development and ageing, is not known (but see Guerin et al (1991) and Pladys et al (1988)). Likewise it is unclear, at this stage, whether modification of sucrose metabolism by pH is a physiologically significant event.

Product inhibition

Sucrose synthase catalyses a freely reversible reaction and so product accumulation, or limitation in the amount of one of the substrates could rapidly alter flux through the pathway. In contrast the reaction catalysed by AI is not reversible and it has been argued that the activity of this enzyme is likely to be under strict control (eg Geigenberger, Stitt, 1993) though there is no evidence for this, as yet. In the direction of sucrose hydrolysis, soybean nodule SS and AI have similar affinities for sucrose (29 and 24 mM, respectively) and both are competitively inhibited by glucose and fructose (SS more strongly than AI). The extent of inhibition of SS and AI will, clearly, depend on the cellular location of the enzymes and also on the proximity and concentrations of their substrates and inhibitors. Recent data on changes in carbohydrate content of soybean nodules during the diurnal period (Gordon unpublished) suggests that glucose and fructose concentrations are c. 10-15 mM and 3-6 mM, respectively. Sucrose concentration, however, varied between 50-55 mM during the photoperiod and declined to a minimum of 12 mM in the dark period. From these calculations it seems likely that SS and AI activities could be seriously affected by hexose concentrations in vivo. It is well documented that the concentration of glucose in nodules is always much higher than that of fructose (eg. Streeter 1987). This is significant and implies that glucose may have a regulatory role, and, since AI is the only means of producing glucose, that AI is involved in this regulation. Equally important are the in vivo activities of HK and FK, since, in conjunction with AI and SS, they will determine the levels of free hexose.

Effect of haem

It was reported by Thummler, Verma (1987), that SS was regulated by free haem in legume nodules. They suggested that haem inhibited SS by causing dissociation of the tetrameric enzyme to inactive monomeric subunits. We confirm that partially purified SS is inhibited by hemin such that only 75, 45 and 28% activity remained when assayed in the presence of 20, 50 and 100 μM hemin, respectively.

In further studies we investigated inhibition of a range of enzymes by hemin in crude extracts. In the presence of 50 μM hemin, PEPC and AI activities were eliminated; G6PDH and MDH were reduced to 25% of control activities, while PGI, PGM and AAT still retained 80-90% of their activity. SS activity was reduced to 75-80% of controls. We conclude, therefore, that free haem could inhibit of a spectrum of nodule enzymes and could seriously affect nodule activity. In animal systems, haem is known to bind to proteins resulting in conformational changes which (a) could cause changes in enzyme activity directly, or (b) expose portions of the protein to proteolytic attack (Shin et al, 1994).

Control at the level of gene expression

In addition to fine control of metabolic steps by interactions with effector molecules to adjust in vivo activity of pre existing enzymes, a longer term control could take place via changes in gene expression or in the turnover of particular proteins (Sullivan, and Green, 1993). To test these possibilities we are using stress perturbations to study the response and sensitivity of legume nodules. A variety of treatments either directly or indirectly affect nodule N_2 fixation and this may be part of an inherent mechanism to balance the needs of the plant as an integrated whole (cf Parsons et al 1993). Control of N_2 fixation may be is exerted by adjustments to the oxygen diffusion barrier (Layzell et al 1993). However, in at least one case, drought stress, the response of legume nodules was not solely due to 0_2 limitation; there is evidence that there is adjustment in the metabolic potential of nodules (Guerin et al, 1990; 1991; Hunt, Layzell, 1993; Diaz del Castillo et al, 1994). Guerin et al (1991) suggested that this was due to a decline in the leghaemoglobin content of *Vicia faba* nodules which might be related to an increase in activity of an alkaline serine protease. In alfalfa root nodules (Irigoyen et al 1992) drought caused increases (transient and longer term) in the specific activities of a number of enzymes. During development of a mild drought stress in soybean, however, we found significant declines in the activity of nodule SS (to 50 and 25% of controls after 4 and 11 days of stress), (Gonzalez et al, 1995). Leghaemoglobin, and other enzymes activities were unchanged. We argue that the decline in SS could have significant effects on the ability of nodules to metabolise sucrose (Gonzalez et al, 1995) particularly if, as reported by Guerin et al (1991), intracellular pH rises during drought stress. In more recent experiments we have investigated a number of other stresses to normal nodule function in soybean (NO_3^-, salt, darkness, drought, shoot meristem removal, partial nodule removal) and find that SS is particularly responsive in treatments which cause a decline in N_2 fixation. In addition, SS transcript levels declined even more dramatically (Gordon, et al, unpublished). Thus the trend is similar to that in dark stressed plants (Gordon et al 1993) where nodule SS mRNA disappeared within 24h, followed by declines in SS protein. Lb and GS mRNA also declined markedly, but no change was found in the amounts of the corresponding proteins. The factors which bring about these changes in nodule gene expression need urgent investigation.

Our conclusions from this work are (1) that gene expression in nodules is rapidly affected by perturbations which affect whole plant stress status and (2) that SS gene expression and SS protein and enzyme activity may be particularly sensitive. The result would be a reduction in the rate of sucrose metabolism and a decline in N_2 fixation, and, simultaneously or subsequently, a plethora of other effects such as changes in phloem import, export of products and the content in nodules of N products, carbohydrates etc which could have further effects on the expression of other nodule genes (Sheen, 1994).

This hypothesis incorporates a direct link between photosynthesis and N_2 fixation, mediated by phloem delivery of sucrose, water (Walsh 1990) and nitrogenous signal molecules (Parsons et al, 1993) and effected via control of the nodule enhanced enzyme, sucrose synthase.

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Malic Enzymes of *Rhizobium meliloti*

DRISCOLL B.T., OSTERAS M., FINAN T.M.
McMaster University, Department of Biology, 1280 Main St W.,
Hamilton Ontario L8S 4K1, Canada.

1. **Introduction:** There is much evidence that C_4-dicarboxylic acids, such as malate and succinate, are the principle energy sources utilized by N_2-fixing bacteroids within root nodules. These acids are taken into the bacteroids via a C_4-dicarboxylate transport (Dct) system which has been extensively studied in a number of *Rhizobium* species (Ronson et al. 1981; Finan et al. 1983; Dilworth, Glenn 1984; Vance, Heichel 1991). In addition to the C_4-dicarboxylates malate and succinate, the possibility that aspartate may also be supplied to bacteroids is supported by the finding that *R. meliloti* mutants lacking a particular aspartate aminotransferase are unable to fix N_2 (Rastogi, Watson 1991). Following transport of the C_4-dicarboxylates into the bacteroids, we believe that they are metabolized via the tricarboxylic acid (TCA) cycle. To our knowledge, all results of enzymatic assays are consistent with the operation of an intact TCA cycle in bacteroids. We are studying the pathway by which the C_4-dicarboxylates malate and succinate are metabolized in bacteroids. Without an efficient pathway for the synthesis of acetyl-CoA, the metabolism of these and other C_4-dicarboxylates via the TCA cycle would lead to a rapid accumulation of oxaloacetate. We have focused on the role of malic enzymes in the generation of acetyl-CoA. Malic enzyme catalyzes the oxidative decarboxylation of malate to pyruvate and CO_2 with the simultaneous reduction of $NAD(P)^+$ to NAD(P)H. The pyruvate thus formed would then be converted to acetyl-CoA by pyruvate dehydrogenase. An additional pathway for the synthesis of acetyl-CoA from oxaloacetate could be mediated by the combined activities of phosphoenolpyruvate carboxykinase (PCK), pyruvate kinase and pyruvate dehydrogenase. The very low PCK activities detected in bacteroids from alfalfa and pea root nodules, together with the symbiotic phenotypes of Pck^- mutants, clearly shows that the PCK pathway is not operative in N_2-fixing bacteroids (McKay et al. 1985; Finan et al. 1991). We note however that Pck- mutants of *R. meliloti* have a reduced N_2-fixation phenotype. In addition, Pck^- mutants of *Rhizobium sp.* NGR234 have a pronounced plant-host-dependent symbiotic phenotype. We attribute the latter two results to a requirement for the PCK enzyme during the infection process, for example growth of the bacteria in the infection thread, rather than a role for PCK during N_2-fixation in the bacteroid (Finan et al. 1991; Østerås et al, 1991).

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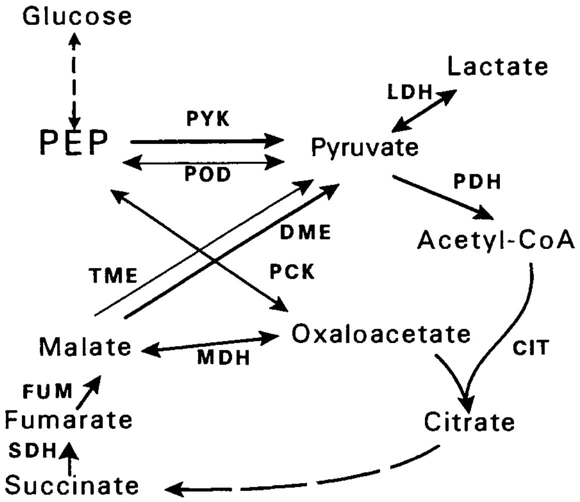

Figure 1. Intermediary carbon metabolism in *R. meliloti*. Abbreviations: CIT, citrate synthase; DME, NAD$^+$-dependent malic enzyme; FUM, fumarase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; PCK, phosphoenolpyruvate carboxykinase; PDH, pyruvate dehydrogenase; POD, pyruvate orthophosphate dikinase; PYK, pyruvate kinase; SDH, succinate dehydrogenase; TME, NADP$^+$-dependent malic enzyme.

2. *R. meliloti* has Distinct NAD$^+$-dependent and NADP$^+$-dependent Malic Enzymes.

Elution of proteins from DEAE-cellulose chromatography columns with a 0-0.3 M KCl gradient revealed two malic enzyme peaks (Driscoll, Finan 1993). Malic enzyme activity in the first peak only occurred in the presence of NADP$^+$, whereas the activity of the peak which eluted at the higher salt concentration showed higher activity with NAD$^+$ than NADP$^+$. For simplicity we refer to these two enzymes as the NADP$^+$-dependent enzyme and the NAD$^+$-dependent enzyme. Each enzyme is encoded by a separate gene and these are referred to as the *tme* gene and the *dme* gene respectively. Malic enzyme activities have also been described for *R. leguminosarum*, *Bradyrhizobium japonicum* and *Rhizobium sp.* NGR234 (Copeland et al, 1989; Kimura and Tajima ,1989; McKay et al, 1988; Østerås et al, 1991)

3. Isolation of NAD$^+$-Malic Enzyme Mutants of *R. meliloti*

While *pckA* mutants grow very poorly on succinate or malate as sole carbon sources, we had observed a class of second-site mutations which allowed *pckA* mutants to grow like the wild-type on succinate and malate, despite the absence of any phosphoenolpyruvate carboxykinase activity. These suppressor alleles were designated *spk* for suppression of PCK. Genetic data suggested that the suppressor mutations resulted in the activation of a PEP synthase-like activity to convert pyruvate to PEP. We therefore reasoned that gluconeogenesis and growth of a *pckA spk-1* strain on TCA cycle intermediates would be dependent on the decarboxylation of malate to pyruvate by malic enzymes, and the subsequent conversion of the pyruvate to PEP by the *spk-1* activated enzyme activity.

Tn5 insertion mutagenesis of *R. meliloti* strain RmG243 (*pckA3 spk-1*) and subsequent screening for growth on TCA cycle intermediates yielded three independent mutants which lacked NAD^+-malic enzyme activity (*dme-1*, *dme-2* and *dme-3*). These three mutations were transferred into the wild-type Rm1021 background and their symbiotic and carbon utilization phenotypes were examined. The Rm1021 *dme* mutants grew like the wild-type on all carbon sources examined, however they formed root-nodules on alfalfa which failed to fix N_2 (Driscoll, Finan 1993). Microscopic examination of these nodules revealed that bacteria were released from the infection threads and were surrounded by peribacteroid membranes. Nodule development thus appears to occur normally up to the stage when N_2-fixation occurs. We interpret these results to mean that the requirement for NAD+ malic enzyme is specific to the N_2-fixation process rather than to some aspect of bacteroid development. In support of the above interpretation, we have observed that nodules induced by *dme* mutants containing a *nifH::lacZ* reporter plasmid showed strong *nifH* expression, as revealed by *in situ* staining for β-galactosidase activity (Finan and Truchet unpublished).

4. *R. meliloti* Mutants lacking NADP+ Malic Enzyme

As described above, in a *pckA spk-1* background, mutations which result in a loss of NAD^+-malic enzyme activity (Dme^-) grow poorly on minimal medium containing succinate or other TCA cycle intermediates as the carbon source. Recombinant pLAFR1 cosmids carrying wild-type DNA which complemented the succinate-growth phenotype of the above mutants were isolated. Analysis of these cosmids allowed us to identify those which carried the wild-type *dme* gene region. In addition, we observed that introduction of the previously isolated cosmid, pRmT100 (Finan et al. 1988, Ronson et al. 1987), into the above *dme* mutants restored partial growth on M9-succinate. When pRmT100 transconjugants were assayed for enzyme activity, they were found to have twice the wild-type level of $NADP^+$-malic enzyme and only background NAD^+ malic enzyme activity. Subsequent studies revealed that pRmT100 carries the *tme* gene in a region distinct from the *rpoN* gene which is also carried on this plasmid (Finan et al. 1988). The *tme* gene region was subcloned and insertions within the *tme* gene were recombined into the chromosome to generate *R. meliloti tme* mutants. The *tme* mutants induced wild-type N_2-fixing nodules on alfalfa. DEAE-cellulose chromatography of protein extracts from these mutants revealed that the $NADP^+$-malic enzyme peak was absent while the NAD^+-enzyme eluted in the same position as in wild-type extracts. As in the case of the *dme* mutations, the *tme* mutations, in an otherwise wild-type background, resulted in no demonstrable change to the wild-type carbon utilization phenotype. However, unlike *dme* mutations, the introduction of *tme* mutations into a *pckA spk-1* background had no effect on the growth of these mutants on succinate. The latter result implies that the malate to pyruvate flux generated by NAD$^+$-malic enzyme cannot be compensated by the NADP$^+$ enzyme. The result also parallels the symbiotic effect of these mutations (*dme*$^-$ Fix$^-$; *tme*$^-$ Fix$^+$), and is thus consistent with the interpretation that the Fix$^-$ symbiotic phenotype of the *dme* mutants results from an inability to generate sufficient acetyl-CoA to allow the TCA cycle to function.

5. spk Alleles Enhance Pyruvate Orthophosphate Dikinase Activity

As free-living phenotypes resulting from the *dme* mutations were only observed in a *pckA spk-1* background, we wished to identify the biochemical mechanism through which the *spk-1* mediated suppression occurred. This task was facilitated by the isolation of three Tn*5* insertion mutations which appeared to map to the *spk-1* locus. These three insertions were isolated in a *pckA spk-1* background and the insertions reversed the *pckA spk-1* growth phenotype to the original *pckA* succinate negative phenotype. We had previously isolated a Tn5-233 insertion, Ω5208, which was 60% linked in transduction to the *spk-1* locus. Southern blot analysis showed that the three Tn5 insertions were located in a 10 kb *Eco*R1 fragment and in transduction these insertions were found to be about 60% linked to Ω5208. As, these results suggested that the three Tn5's had inserted into the *spk-1* locus; the three alleles were designated *spk5*::Tn5 - *spk7*::Tn5. We cloned the *spk-1* locus as part of a pJB3JI derived R' which was selected using the Gm^{r}Sp^r of the Ω::5208::Tn5-233 insertion. Introduction of the R' into *R. meliloti pckA* mutants allowed them to grow on M9-succinate and the same result was obtained with a pRK7813 derivative carrying a 10 kb EcoR1 fragment from the R'. In further experiments, we cloned the transposon insertions, together with the flanking DNA, from the *spk-5* and *spk-6* insertion alleles. Employing an IS50 primer we determined the DNA sequence from one side of each of the insertions. A search of GenBank with this sequence revealed clear homology to pyruvate, orthophosphate dikinase (POD). POD (E.C. 2.7.9.1) catalyses the reversible reaction: Pyruvate + ATP + Pi <—> PEP + AMP + PPi. To measure the POD activity we used an assay in which the AMP and PPi dependent formation of pyruvate from PEP was measured in a coupled assay employing lactate dehydrogenase and NADH. Independently prepared extracts from a strain carrying the *spk-1* mutation had ten fold higher activity than extracts prepared from the wild-type, in which an activity of 2.4 nmoles/min/mg protein was detected. Extracts from strains carrying the *spk-2*, the *spk-3* and the *spk-4* alleles were two-fold, twenty three-fold, and six-fold higher respectively than the wild-type activity. Moreover, there was no POD activity detected in cell-free extracts prepared from the *spk*::Tn5 insertion mutant, RmG274. All of these extracts showed similar malate dehydrogenase activities. The DNA sequence data and the results of POD enzyme assays, together with the genetic data, led us to conclude that the *spk* mutations result in an increase in activity of the POD enzyme.

Thus malic enzyme, together with the increased POD activity, converts sufficient malate to PEP so that the normal route for the formation of PEP from TCA cycle intermediates (i.e catalyzed by PCK) is no longer required. We believe that the leaky growth of *pckA* mutants in media with succinate as carbon source is caused by a low POD activity. We have found that the introduction of the *spk:5*:Tn5 insertion mutation into a *pckA* background eliminates the background growth. Finally, wild-type strains carrying *spk*::Tn5 insertions show no detectible alteration to the wild-type carbon utilization phenotype and nodules formed by these mutants fix N_2 at rates indistinguishable than the wild-type.

We do not know whether the *spk* mutations increase the expression of the *pod* gene or increase the activity of the POD protein. The role of POD in the metabolic machinery of *R. meliloti*, if any, is not apparent. In C_4-plants, POD is an important enzyme in the production of PEP during photosynthesis and in some microorganisms POD replaces pyruvate kinase (Edwards et al. 1985; Wood et al. 1977). In *R. meliloti*, it is clear that the POD enzyme plays only a minor role in the gluconeogenic direction. It is possible that this is a regulatory design for channelling the bulk of gluconeogenesis through the regulated PCK pathway.

6. Source of Reductant for Nitrogenase

Nitrogenase requires both ATP and reductant to convert N_2 to ammonia. In N_2-fixing bacteroids, the ATP is synthesised via oxidative phosphorylation while the source of reductant is unknown. In *Klebsiella pneumonia* the *nifJ* gene product, pvruvate:flavodoxin oxidoreductase, reduces the *nifF* encoded flavodoxin which in turn transfers electrons to nitrogenase (Hill, 1992). How C_4-dicarboxylate metabolism in bacteroids is coupled to the generation of reductant for nitrogenase is currently unknown. In view of the requirement for NAD^+-malic enzyme for N_2-fixation, it will be interesting to determine if a pyruvate:flavodoxin oxidoreductase-like enzyme is present in *R. meliloti* bacteroids.

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REGULATION AND FUNCTIONAL ANALYSIS OF GLUTAMINE SYNTHETASE GENES IN LEGUMES.

TEMPLE S. J., BAGGA S. AND SENGUPTA-GOPALAN C.
Dept. of Agron. & Hort., New Mexico State University, Las Cruces, NM 88003, USA.

INTRODUCTION

Glutamine synthetase (GS) is the key enzyme responsible for the assimilation and reassimilation of ammonia. GS catalyzes the ATP dependent condensation of NH_3 with glutamate to produce glutamine. Subsequently, glutamate synthase transfers an amido group of glutamine to $\propto$-ketoglutarate to produce two molecules of glutamate (Lea et al., 1990). Glutamine is one of the major nitrogen transport compounds in plants and acts as the nitrogen donor for the synthesis of most nitrogenous compounds. GS in plants, is made up of eight subunits, each subunit having a molecular weight of between 38-45kD. The GS isoenzyme localized in the plastid/chloroplast (GS_2) functions in the reassimilation of NH_3 produced by photorespiration, while the cytosolic isoforms of GS (GS_1), depending on the organ/tissue where it is located, assimilates ammonia produced by different physiological processes (McGrath, Corruzzi, 1991). The GS_1 in roots, stems and leaves is localized around the vasculature and it is believed to function in generating glutamine for nitrogen transport (Brears et al., 1991). The GS_1 in the root vasculature is believed to be involved in the assimilation of ammonia produced by the reduction of nitrate obtained from the soil water. In root nodules, GS_1 is localized mostly in the infected cells where it assimilates ammonia produced by the N_2-fixing bacteroids.

GS_1 in higher plants is encoded by a small multigene family and the different members show differential patterns of expression (Hirel et al., 1993). In alfalfa, two subclasses of GS_1 gene have been identified that share high sequence identity in the coding region, but have unique 3' untranslated regions (3'UTR) (Temple et al., 1995). To understand the functional significance of the two subclasses of GS_1 genes we have used the antisense RNA technology (Bourque, 1995) to downregulate GS_1 expression in a subclass specific and vasculature specific manner. We have also introduced gene constructs into alfalfa plants aimed at upregulating GS_1 levels in a constitutive manner.

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Results

Identification of GS_1 subclass specific polypeptides: RNA from a few different organs of alfalfa plants was subjected to hybrid select translation (HST) using the subclass specific 3'UTR (pGS100 and pGS13) followed by 2D SDS-PAGE. The HST products were co-electrophoresed with the protein extract from the same organ. The fractionated proteins were electroblotted onto PVDF membrane and the membrane subjected to western analysis followed by autoradiography. The primary translation products have a corresponding immunoreactive polypeptide, implying that the primary translation products do not undergo any post translational modification. Analysis of the HST product profile along with the GS_1 polypeptide profile suggests that each of the two subclasses has two members each, a1 and a2 (pGS13) and b1 and b2 (pGS100). However, the gene member a1, is represented by two allelic forms, a1/a1' which differ slightly in their pI values. The polypeptides a2 and b1, comigrate as expected, based on the predicted molecular weight and pI values for the pGS13 and pGS100 gene products (39.2 and 5.39 respectively). This was further confirmed by analyzing the 2D gel profile of GS polypepetides from transgenic tobacco constitutively expressing either pGS100 or pGS13 genes.

Expression profile of the pGS100 and pGS13 genes in the different organs: To understand the functional significance and the regulatory mechanism underlying the expression of the two subclasses of GS_1 genes in alfalfa, RNA from different plant organs were subjected to northern analysis using the pGS100 and pGS13 3'UTR as probes. The two probes hybridized to RNA from all organs though at significantly different levels. The nodules showed the highest level of hybridization with the pGS13 3'UTR probe followed by the stem. The pGS100 3'UTR probe showed the highest level of hybridization to RNA from mature roots followed by nodule RNA.

Sub-class specific downregulation of pGS100 genes in alfalfa: In an attempt to distinguish between the functional roles of the pGS100 and pGS13 encoded gene products, a gene construct aimed specifically at downregulating the pGS100 gene (pGS150) was introduced into alfalfa. The gene construct pGS150 consists of the 3' untranslated region of pGS100 in antisense orientation fused downstream to the hygromycin phosphotransferase gene under the control of the CaMV 35S promoter. The transformants showed up to 75% reduction in pGS100 related transcripts, the reduction being the most dramatic in the nodules, with no significant changes in the levels of pGS13 transcripts. Interestingly, the same transformants showed an increase in total GS_1 polypeptide level and GS activity in the stem, roots and nodules. Western anaylsis of protein fractionated by 2D SDS-PAGE showed that the spot corresponding to a2 and b1 is significantly increased in the stem and nodule and that there is a novel new protein (labeled 'c') which appears to be synthesized specifically in the pGS150 transformed alfalfa (Fig 1). To see if the synthesis of the GS_1 polypeptide 'c' is a result of transcriptional activation, RNA from the stem of a transformant and a control plant was subjected to hybrid selection using the coding region and the 3'UTR of both pGS100 and pGS13. The hybrid selected RNA was translated *in vitro* and the translation products subjected to 2D SDS-PAGE. Comparison of the HST products showed that a spot corresponding to polypeptide 'c' was present only in the transformant. The mRNA for the polypeptide 'c' was not selected by the 3'UTR of either pGS100 or pGS13.

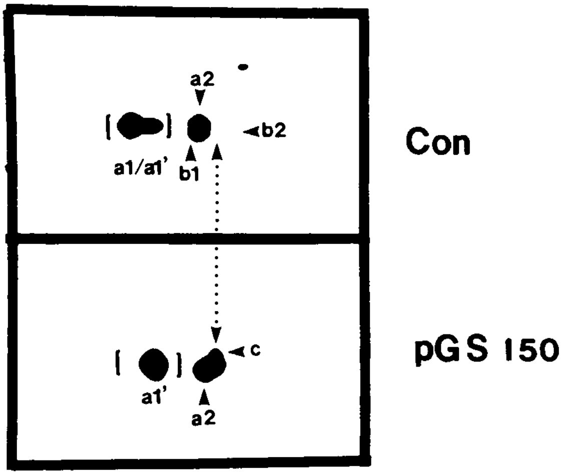

Figure 1. *Comparison of the nodule GS profile between control alfalfa and a pGS150 transformant alfalfa plants.* Total soluble protein from nodules (10mg) were fractionated by two dimensional SDS-PAGE and probed with GS antibody. The arrowheads point to the different GS_1 polypeptides while the dashed line with arrowheads show the position of the new GS_1 polypeptide in the transformant.

Downregulation of GS_1 in a vasculature specific manner: We also introduced an antisense GS_1 construct specifically aimed at downregulating GS_1 transcripts in the vasculature. The acidic chitinase promoter from *Arabidopsis* which functions in a vasculature enhanced manner in tomato (Samac, Shah, 1991) and *Arabidopsis*, was used to drive the pGS100 cDNA sequence in an antisense orientation. Based on GUS staining of transgenic alfalfa transformed with the acidic chitinase promoter fused to GUS, the vasculature in the nodules showed the most intense staining. A cross section of the stem showed that in the vascular tissue, the phloem was the most heavily stained. The construct pGS220 (acidic chitinase promoter with pGS100 in antisense orientation) was introduced into alfalfa. The resulting transformants were pale yellow in tissue culture with a gradual decrease in pigmentation starting from the midvein to the outside and showed a high degree of lethality. Only a few transformants survived and grew into mature plants. Young growing tissues of these transformants continued to show characteristic yellowing which gradually greened up as the tissues matured. Analysis of the mature transformants showed that there was a dramatic reduction in both pGS100 and pGS13 transcripts in all the organs tested. At the GS_1 protein level, except for the stems all the other organs showed a slight decrease in the level of GS_1 polypeptide. The stem of the pGS220 transformants, however, showed a significant increase in the GS_1 polypeptide level when compared to the control. (Temple et al., 1994).

Constitutive over expression of the pGS100 gene: The pGS100 cDNA engineered in the sense orientation behind the CaMV 35S promoter (pGS111) was used to transform alfalfa. The transformants showed no significant increase in GS_1 polypeptide levels in the leaves but did show a significantly higher level of GS_2 polypeptide when compared to control plants (Fig 2, panel C). To better understand the basis for increased GS_2 polypeptide levels without any significant changes in GS_1 levels in the pGS111 transformed alfalfa plants, we checked the levels of the corresponding transcripts in these plants. RNA from three of the alfalfa transformants and two control plants was subjected to northern analysis using pGS100 3'UTR, pGS13 3'UTR and a pea GS_2 cDNA (kindly provided by Dr. Gloria Coruzzi) as probes (Fig 2, panel B). Since the transgene uses the NOS 3' terminator, the transcript corresponding to the transgene is slightly bigger than the endogenous pGS100 transcript. The transformants showed up to five fold higher level of transcript compared to the endogenous pGS100 transcript level in the control plants. No significant difference was found in the pGS13 and GS_2 transcript levels between the transformants and the control.

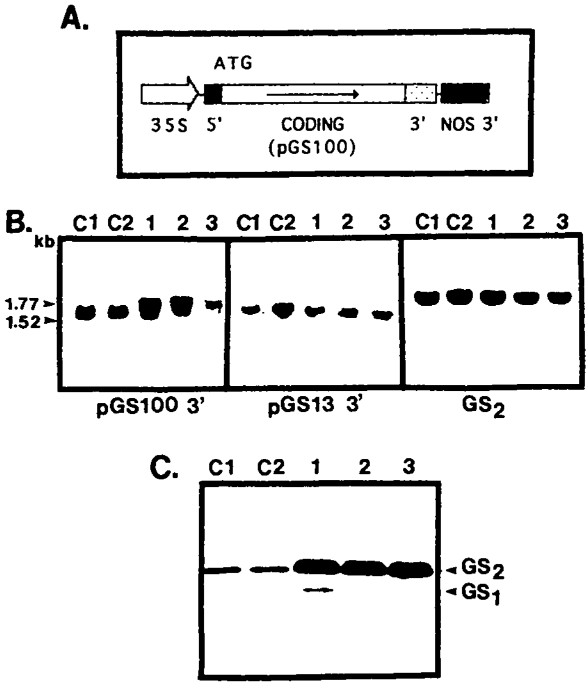

Figure 2. *Analysis of pGS111 transformed alfalfa plants.* Three transformants (labeled 1, 2 and 3) and two control plants (c1 and c2) were subjected to northern and western analysis. Panel A: description of the gene construct; Panel B: Northern blots were probed with pGS100 3'UTR, pGS13 3'UTR and a GS_2 cDNA probe; Panel C: Western blots showing GS_2 and GS_1 polypeptides.

DISCUSSION

We previously classified the alfalfa GS_1 genes under two broad subclasses based on sequence homology in their 3'UTR region (pGS100 and pGS13 related gene subclass)

(Temple et al., 1995). The data presented here suggests that there are two pGS100 and two pGS13 related gene members, one of which has two distinct alleles (a1/a1'). While both gene subclasses are expressed in all organs, there are organ specific differences in expression pattern of the two gene subclasses, suggesting differential regulation.

Alfalfa transformants containing an antisense gene construct specific for the 3'UTR of pGS100, showed a significant drop in pGS100 hybridizable transcripts with no significant change in the level of pGS13 related transcripts. This would suggest that downregulation due to the antisense construct is specific for pGS100 related genes. It is, however, difficult to assess the extent to which protein b1 is downregulated since it comigrates with the pGS13 related protein a1. However, a closer examination of the 2D western profile of the pGS150 transformant when compared to the control, indicates a change in the shape of the spot corresponding to b1 and a2 (Fig 1). This could be due to a decrease in the spot corresponding to b1 and an increase in a1. Thus downregulation of b1 appears to be accompanied by an increase in polypeptides corresponding to pGS13 genes. Since there is no change in pGS13 transcripts it would imply that the increase in the protein level is regulated at the level of translation or protein stability. The appearance of a new GS_1 polypeptide in the nodules and to some extent in the stem of the pGS150 plants suggests that in these organs some of the compensation is at the level of gene induction. Analysis of the HST products confirms that the induction is at the level of transcription or transcript stability. This novel gene encoding for polypeptide 'c' does not have the same 3'UTR as the pGS13 or pGS100 genes since transcript was not selected by either of the 3'UTR. This gene is normally silent, but is probably induced under conditions of high substrate availability.

Our experiments utilizing the acidic chitinase promoter suggest that downregulation of the GS_1 genes in a vasculature specific manner is lethal. The fact that the three pGS220 tranformants that survived showed up to 80% reduction in pGS100 and pGS13 related transcripts, suggests that the plants require only a low level of the GS_1 transcripts in the vasculature for survival. Moreover, inspite of a decrease in the transcript level, the stems actually showed an increased GS_1 polypeptide levels, providing further evidence for some kind of compensation probably at a translational or post-translational level.

The most intriguing of all the results is that overexpression of the pGS100 genes in a constitutive manner in alfalfa does not show the expected increase in the GS_1 polypeptide level in the leaves or stem. Moreover, it appears that these transformants showed a downregulation of the endogenous pGS100 genes with no change in the level of pGS13 or GS_2 transcripts. We propose that in the absence of sufficient concentrations of the substrates, NH_3 and/or glutamate, GS enzyme is unstable and turns over. It is also possible that glutamate synthase is limiting under conditions of constitutive overexpression of GS.

Taken together, our data suggest that the GS_1 gene member encoding for 'c' is regulated by either substrate availability, or by the ratio of glutamine to ∝-ketoglutarate. Metabolite control of GS expression in bacteria and yeast is well documented (Wood, Reid, 1993; Benjamin et al, 1989). An ammonia inducible GS_1 gene of soybean has been characterized (Miao et al., 1993). We further propose that the assembly of GS subunits or stability of the assembled enzyme is dependent on the availability of substrates, thus explaining the low level of GS_1 polypeptide accumulation in the leaves of pGS111 transformed alfalfa plants. Additional evidence for this proposal comes from our work on GS_1 expression in soybean nodules. We have shown that in nodules formed by Nif^-strains of *B. japonicum*, even though the nodule specific GS_1 genes are transcribed and translated normally, the subunits are unable to assemble into a stable holoenzyme as in the case of nodules formed by WT strains of *B. japonicum* (Temple S.J., unpublished date). Work is in progress to test the effect of downregulating the pGS13 related genes in a subclass specific manner and to overexpress GS_1 in a nodule specific manner.

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ROLE OF THE PERIBACTEROID MEMBRANE IN THE INTERACTIONS OF THE SYMBIOTIC PARTNERS IN THE NITROGEN-FIXING ROOT NODULES OF *LUPINUS LUTEUS* L.

KUDRYAVTSEVA N.N, TSYDENDAMBAEV V.D., DUBROVO P.N. KRYLOVA V.V., BORODENKO L.I., ZHIZNEVSKAYA G.YA., IZMAILOV S.F.
K.A.Timiriasev Institute of Plant Physiology, Russian Academy of Sciences, Moscow

In legume root nodules, symbiotic *Rhizobium* are separated from the host cell cytosol by the peribacteroid membrane (PBM). The PBM appears to play an essential role in the interaction between two partners of symbiosis. During the early stage of senescence, or when nodulated plants are submitted to some stress, the PBM is one of the first structures to be degraded and its disappearance parallels the loss of nitrogen-fixing activity; indeed, loss of the PBM could well participate in the senescence process and even provoke it. However, PBM biogenesis, the changes of PBM structure, its unctioning in the course of development, and the operation of the symbiotic system have never been completely documented. This involves the role of protein-lipid complexes of the PBM in symbiont interrelations.

In this work, we have studied the activity of H^+-ATPase bound with PBM and the relative fluidity and fatty acid composition of the lipid bilayer of PBM isolated from effective and ineffective root nodules of the *Lupinus luteus* L. The possible influence of symbiosis effectiveness on the PBM state is discussed.

MATERIALS AND METHODS

Yellow lupin (*Lupinus luteus* L.) plants were grown in a sand-containing vessels in greenhouses. Plants were inoculated with *Bradyrhizobium sp.* (*Lupinus*) effective strain 359a and spontaneously noneffecive strain 400. The latter is characterized early senescence and easy rapture of PBM (Andreeva, 1985) and more heterogeneity of the total membrane. Peribacteroid embrane vesicles and membrane fraction enriched with plasmalemma were obtained by the methods described earlier (Izmailov et al., 1989, Genina et al., 1993). Transmembrane proton transport on freshly isolated vesicles was studied on a Hitachi-557 spectrophotometer from changed in absorption of Acridine orange (AO) in a two wave regime (A492-540). Determination of ATPase activity was based on the increase of inorganic phosphate over 30 min at 30°C. Fatty acids were identified according to the relative retention times of their esters compared to this of methylpalmitate, and by comparing with authentic standards. The concentration of each fatty acid in the mixture was expressed as a weight per cent of the total.

Membrane fluidity was estimated from steady-state fluorescence anisotropy using 1,6-diphenyl-1,3,5-hexatriene (DPH) probe. Polarization measurements of DPH fluorescence were performed under room temperature with a MPF-4 fluorescent

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spectrophotometer (Hitachi, Japan). All experiments were repeated either four or five times.

RESULTS AND DISCUSSION

A common feature of the endosymbiotic association is the presence of H^+-translocating enzyme system on the membrane. These enzymes is, in the first place, are ATPases. Their activities result in the energization of symbiotic membrane and acidification of PBS. H^+ATPase is the key enzyme system of plasma membrane in higher plant cells (Sze, 1985). It is involved in primary and secondary active transport of ions and metabolites. According the Durbrovo's data (Dubrovo et al., 1992) it can be assumed that Mg^{2+}-ATP-dependent transport of protons into PBM vesicles was clearly manifested using pH indicator AO. The proton gradient created was completely removed by addition of the protonophore 2mM gramicidin D, the H^+/K^+-antiporter nigericin, or the penetrating cation NH_4^+ (10mM NH_4Cl). This means that obtained preparations of PBM vesicles contained H^+- ATPase with active site located on the outer side. Formation of a pH gradient occurred only when the incubation medium contained a penetrating counter ion. When KCl was excluded from the incubation medium, the rate of proton transportdeclined sharply and did not increase upon the addition of K_2SO_4 . However, it increased significantly again on addition of KCl or NaCl . The process of acidification of intracellular space took place more slowly with another penetrating anion, NO_3^- .

Investigation of the proton transport rates in relation to pH in the interval of 4.5-8.8 indicate the presence of a peak at the pH 5.7 with preservation of about 50% of maximum activity at pH 6.5-7.5. (Dubrovo et al., 1992). The rate of the hydrolysis of Mg^{2+}-ATP in the presence of sodium azide - an inhibitor of mitochondrial ATPases - attained the maximum in the region of pH 5.7 which is corresponded to the peak H^+-ATPase activity. It was shown that there is a very close correlation between changes in the phase-structural state of the membrane lipid matrix and changes in the hydrolytic and electrogenic activity of the membrane H^+-ATPase. The sequence of events in the plasmalemma and leading to the modulation of H^+-ATPase activity through changes in the fluidity of the membrane lipids can be represented in the following therms: phase-structural reorganizations in the lipid matrix; decline of the lipid fluidity; decrease in the activity of H^+-ATPase or vise versa (Opritov et al., 1992). Fluorescence anisotropy of membrane isolated from the effective (PBM fix^+) and ineffective (PBM fix^-) root nodules and plasmalemma (Pl) from uninfected lupin roots was investigated. We used the steady-state fluorescence anisotropy of a lipophilic probe DPH to estimate the structural state of the PBM hydrocarbon layers (Table 1).

It can be seen that fluorescence polarization values were significantly higher in DPH-labeled PBM (fix^+) as compared with PBM (fix^-). The highest values were in the case of DPH-labeled plasmalemma (Pl). The high values observed (0.2-0.25) for fluorescence polarization of DPH incorporated into these membranes was characteristic, as it was supposed, for gel (rigid) phase membrane. The low values of ca. 0.15 for fluorescence polarization of DPH were characteristic for the liquid phase.

Table 1. Effect of temperature on the values of DPH fluorescence anisotropy (r) of the PBM from effective and noneffective lupin root nodules and from the plasmalemma.

Temperature,^{0}C	PBM fix$^+$	PBM fix$^-$	Plasmalemma
5	0.270	0.248	0.255
10	0.262	0.240	0.251
15	0.258	0.225	0.250
20	0.248	0.200	0.250
30	0.221	0.168	0.248
35	0.210	0.160	0.232
40	0.200	0.150	0.225
50	0.170	0.145	0.170
60	0.150	0.140	0.160

Similarly, temperature-dependent values of the DPH- fluorescence an anisotropy were higher for PBM(fix$^+$). Corresponding curves for PBM (fix$^+$) and PBM (fix$^-$) displayed a single break point in the slope of polarization profile at temperature of approximately 20°C for PBM from effective root nodules and 10°C for ineffective ones. At the temperature below the break point the PBM is in a transition phase state, an intermediate state between gel and liquid phases. This transition phase was more broad than in the case of PBM(fix$^+$). Hence, the PBM (fix$^-$) has a more disorder degree than PBM(fix$^+$). Labeled PBM (fix$^+$) showed the increased r values reflecting more rigidity of the bulk lipid bilayer. The decrease of r, as in the case of PBM (fix$^-$), indicates a substantial increase of liquid phase of a bulk lipid bilayer and shows a heat-induced increase in membrane fluidity.

Since DPH is located in the hydrocarbon layer of the membrane at the level of the 10th-12th carbon atoms of fatty acid chains, it is unlikely that decline of r was associated with the increase in the number of double bonds of fatty acids of the lipide matrix of their PBM. Therefore it allows us to speak about the dependence of r mainly on the solid/liquid phase domains ratio. It was possible to postulate that the r values for membranes are associated with the density of packing and orderliness of lipids in their lipid matrix. Therefore the protein/lipid interaction will be strengthened with the increase in the concentration of transmembrane proteins. The changes the protein composition also influence on the lipid status and can lead to alteration in these order. Anisotropic properties (which are determined by molecular packing of the membrane lipid matrix) are, therefore, higher in the case of PBM (fix$^+$) than in PBM (fix$^-$). The changes in the protein composition in the case of PBM from noneffective root nodules (Agibetov et al., 1992) can be a reason of the alteration in the lipid/protein package and lead to formation of more disordered state of the lipid matrix.

Increased bilayer rigidity in PBM (fix$^+$) will result in increased passive H$^+$-permeability only if it is reflective of a phase transition. It is conceivable that the inflection in temperature plots of polarization for PBM could be attributable to physical changes in the lipid bilayer. The usual interpretation of this phenomenon is that discontinuities originating at the boundaries of the rigid and fluid (disordered) domains in the bilayer give rise to pores through which the solutes pass. H$^+$- ATPase works in a reversible manner; the direction of the reaction is determined by the DmH$^+$ value (transmembrane difference in electrochemical potentials of H$^+$ ions). If DmH$^+$ is low because of high H$^+$ conductivity of the membrane, H$^+$- ATPase hydrolyses ATP, dissipating the cell's energy resources Chernyak et al., 1986). A change in the PBM fluidity during the active N_2-fixation could be a mechanism by which more rigidity of PBM enhances the passive H$^+$ conductance and, indirectly, inhibits H$^+$ gradient generation by ATPase. On the early stage of active N_2-fixation, when the cytosol has al alkaline pH, H$^+$- ATPase with pH optimum 6.5-.7.5 works and its activity consist of only 50% from the activity of acid ATPase (pH 5.7) (Dubrovoet al., 1992). When the conditions change (cytosol pH became more acid, the lipid matrix of PBM is more liquid) the more active H$^+$-ATPase with pH optimum 5.7 begins to work. On the other hand, increased of liquid phase of the bulk lipid matrix bilayer is related to its more accessibility for heme interaction (Zhiznevskaya et al., 1994).

In Table 2 the fatty acid composition of PBM, Pl and free-living rhizobia is presented. The fatty acid composition of PBM and Pl shows a qualitative similarity, but differs in quantitative respect. In the PBM (fix$^-$) lipides 16:0 and 18:1 acids predominate (> 60%). All membranes studied here contain a mixture of straight-chain saturated and unsaturated fatty acids with carbon chains of 14 to 18 atoms, but PBM (fix$^-$) includes also branched chain ones. One can see that lipid fatty acid composition in the membranes of the systems of different N_2- fixing ability are different. The content of α-linolenic (all cis,18:3) and oleic (cis-9-18:1) acids is appreciably higher in the PBM (fix$^+$), than in the PBM (fix$^-$) and Pl and the f fatty acids of bacterial origin (the fatty acid with branched chains and 17:0cyc) are absent in its lipids. At the same time the level of C16-fatty acids is lower in the PBM(fix$^+$) in the comparison with PBM (fix$^-$) and is just the same in the Pl. The content of C18:0 (stearic acid) is lower in PBM(fix$^-$) than in the PBM(fix$^{+)}$ and Pl whereas the content of linoleic acid (C18:2) is similar in all cases. It is known that acyl chain order (proximity of packing within the bilayer) and bilayer thickness increase with the acyl chain length and saturation degree, viz. saturated > trans-unsaturated > cis- unsaturated. And in this respect the PBM (fix$^+$) has a slightly lower lipid bilayer thickness than PBM (fix$^-$) bilayer.

An important feature of fatty acid composition of free- living *B.sp.(Lupinus)* 400 (fix$^-$) is the presence of branched chain fatty acids (ca. 40% of total) which are absent in the fatty acid composition of the PBM (fix$^+$) and the plasmalemma. From the table 2 it is also follows that the total membranes of *B.sp.(Lupinus)* 359a strain composed mainly with the normal straight-chains fatty acid. rhizobia strains which form PBM including significant part of bacterial lipids appear to form ineffective symbiosis. The influence of the rhizobia strain on the effectiveness of symbiosis was confirmed by the PBM fluidity data (Table 2), because the higher unsaturation index (UI) and, consequently, the higher fluidity results in the broader temperature region in which membrane-bound enzymes could work.

Table 2 . Fatty acid composition of the PBM isolated from effective and ineffective lupin root nodules, the plasmalemma (Pl) isolated from uninfected lupin roots and total membranes of free-living *Bradyrhizobium sp. (Lupinus)* strain 359a (fix$^+$) and strain 400 (fix$^-$). Values are expressed as weight per cent of total fatty acid.

Fatty acid	Plasmalemma	Peribacteroid membrane		*Bradyrhizobium sp.(Lupinus)*	
		fix$^+$	fix	359a	400
i14:0	-	-	2.1	-	5.8
14:0	2.3	1.4	2.3	1.0	4.0
i15:0	-	-	-	-	8.3
15:0	1.5	0.8	1.1	-	2.8
ai15:0	-	-	-	-	33.1
i16:0	-	-	-	-	3.3
16:0	37.2	39.2	43.1	9.5	17.9
16:1(n-7)	3.1	3.1	2.3	-	-
16:1(n-9)	-	-	5.7	-	-
17:0cyc	-	-	0.9	-	0.1
18:0	6.3	4.3	1.1	2.4	7.8
18:1(n-7)	-	-	8.1	37.1	5.4
18:1(n-9)	11.8	23.4	11.8	20.9	8.6
18:2	9.5	11.5	13.0	1.3	3.1
18:3	10.7	17.1	3.9	-	-
18:2/18:3	0.9	0.67	3.30	-	-
18:2/16:0	0.29	0.30	0.30	-	-
UI	0.56	1.00	0.77	-	-

In our study we have calculated the 18:2/16:0 and 18:2/18:3 ratios as the additional indexes of fatty acid composition of the PBM lipids. In our case 18:2/16:0 ratio in all samples investigated was practically the same (Table 1). Hence, it concluded that this index of fatty acid composition used by some authors doesn't depend on the effectiveness of symbiosis in the lupin root nodules. The ratio 18:2/18:3 was highly variable in all membrane studied (Table 2) and it was connected with the different 18:3 level which depends on the N_2- fixation effectiveness.

In our opinion, the principle mentioned above is not universal for all macro- and microsymbiont pairs. As in the case of the pair *Vicia faba* L. and *Rhizobium leguminosarum* strains 87(fix$^-$) and 97(fix$^+$) we have found another picture, because in this pair 18:2/16:0 ratio depends on the symbiosis effectiveness (1.4 and 0.3 for PBM fix$^+$ and PBM fix$^-$, respectively), but 18:2/18:3 ratio in both cases was almost the same (4.2 and 3.7, respectively). In the case of *Vicia faba* L. symbiosis the 18:3 level was low (2-6% of total fatty acids) and the increase of fluidity in the effective symbiotic pair is connected with the decrease of 16:0 and the increase of 18:2. However, we can suppose that also in this case the effectiveness of symbiosis is determined by the ability of rhizobia and/or symbiont pair to form PBM mainly from the plant host lipids.

On the basis of the data obtained it can be seen that noneffective rhizobia lipids contains both typical bacterial fatty acids (branched-chain, cyclopropanoid, etc.) and normal straight -chain fatty acid. Both of that may take part in the PBM formation, but there is some threshold level of these lipids in the PB matrix to allow the symbiosis to be effective. If the level of branched-chain fatty acid is exceeded the symbiosis becomes ineffective.

During the investigation of total of fatty acid mixture isolated from PBM, bacteroid and bacterial total membrane we have found the peak with Rt = 16.5 as well. According to preliminary identification this peak is corresponded to methyl ester derivative of poly-β-hydroxybutyrate, which is normally present in rhizobia and probably associated with PBM. It is presented also on the bacteroid membrane, but in a less degree. In our opinion, it is the another evidence of the *Rhizobium* participation in the process of the PBM formation.

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NITROGEN AND CARBON EXCHANGE ACROSS SYMBIOTIC MEMBRANES FROM SOYBEAN NODULES.

DAY D.A.(1), WHITEHEAD L.(1), HENDRIKS J.H.M.(1),
TYERMAN S.D.(2).
(1) Division of Biochemistry and Molecular Biology, Faculty of Science,
Australian National University, Canberra, ACT 0200;
(2) School of Biological Sciences, The Flinders University of South
Australia, GPO Box 2100, Adelaide 5001, Australia.

Metabolites exchanged between plant and bacteroid in nitrogen fixing legume nodules must traverse two permeability barriers (the plant-derived peribacteroid membrane - PBM - and the bacteroid membranes) which have different polarities and which delineate three compartments of different pH (see Fig. 1). The ATPase on the PBM pumps protons into the peribacteroid space (PBS) leading to an electrical potential ($\Delta\psi$) positive on the inside (Udvardi et al., 1991); the respiratory electron transport chain of the bacteroid also pumps protons into the PBS, creating a $\Delta\psi$ of the opposite polarity. Together, the PBM and bacteroid pumps ensure that the PBS is acidic relative to the plant and bacteroid cytoplasms (Fig. 1). These properties will influence the nature of ionic movements between the symbionts. Carriers and channels exist on the two sets of membranes which utilise the energetics of the systems to catalyse rapid transport of reduced carbon into, and fixed nitrogen out of, the bacteroid. This paper reviews our current knowledge of these transporters in *Bradyrhizobium*-legume symbioses.

Isolation of symbiosomes

Identification of transport mechanisms on the PBM is most conveniently performed with isolated symbiosomes (the peribacteroid unit consisting of bacteroids enclosed within the PBM). To date most of these studies have used nodules in which several or many bacteroids are enclosed in large symbiosomes: these include soybean, siratro and french bean (Day et al., 1989; Herrada et al., 1989; OuYang and Day, 1992). In studies such as these, is imperative that the purity and integrity of symbiosome preparations are ascertained to avoid misleading results, since even minor contamination by free bacteroids can lead to erroneous conclusions (Udvardi et al., 1988b and Herrada et al., 1993). One advantage of using large symbiosomes is that their high density allows them to be readily and quickly purified by centrifugation on percoll gradients, avoiding contamination by plant organelles which can confuse transport studies. Large symbisomes are somewhat fragile but their integrity can be easily checked by light microscopy (Day et al., 1989) and can be monitored during uptake experiments by measuring their volume which is much larger than that of free bacteroids (Ou Yang et al., 1990). The integrity of the PBM in

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soybean and french bean symbiosome preparations can also be determined by the rate of glutamate uptake. The PBM is impermeable to glutamate in these systems, while bacteroids possess an active transporter (Udvardi et al., 19988b; Herrada et al., 1989; OuYang and Day, 1992): thus PBM rupture leads to an increase in the rate of glutamate uptake by the symbiosome preparation (Herrada et al., 1993). The density of large symbiosomes also allows them to be separated from any contaminating free bacteroids if the centrifugal filtration technique (Palmieri and Klingenberg, 1979) is used to measure uptake.

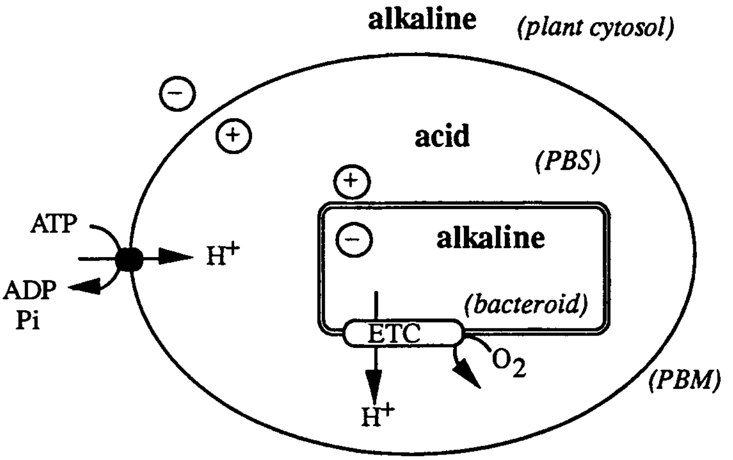

Figure 1. Styalised drawing of a symbiosome, illustrating the membrane polarity and pH gradients between compartments. PBM: peribacteroid membrane; PBS: peribacteoid space; ETC: bacteroid electron transport chain.

Carbon transport into symbiosomes

Dicarboxylate transport

Evidence for dicarboxylates as the major source of reduced carbon supplied to the bacteroid is now overwhelming (see Streeter, 1991 and Vance and Heichel 1991 for reviews). In addition to metabolic and genetic evidence (O'Garaet al., 1988), all symbiosomes so far studied have a dicarboxylate carrier on their PBM (OuYang et al., 1990; Herrada et al., 1989; OuYang and Day, 1992; Rosendahl et al., 1992). This carrier has a preference for malate and succinate,with the former the most likely candidate *in vivo*.

Malate transport into soybean symbiosomes is driven by the $\Delta\psi$ across the PBM and also by its sequestration in the bacteroid (OuYang et al., 1990), and is stimulated by phosphorylation of PBM proteins via a protein kinase on the membrane (OuYang et al., 1991). The most prominent protein phosphorylated on the PBM is nodulin 26 (Weaver et al., 1991) and it is possible that this protein is itself the dicarboxylate carrier, since isolated nodulin 26 acts as a channel in lipid bilayers (Weaver et al., 1994). However, its selectivity for ions is poor and further evidence is required to confirm its identity. It should be noted that control of PBM dicarboxylate transport by protein phosphorylation has only been demonstrated in soybeans; in french bean symbiosomes, no 26 kDa protein is phosphorylated in the presence of ATP and stimulation of the carrier by ATP is transient, disappearing upon removal of ATP (S. Moreau, A. Puppo and D.A. Day, unpublished results).

Bacteroids isolated from various legumes possess a dicarboxylate carrier which has been studied extensively at the molecular genetic level (Ronson et al., 1981). However, little is known about its mechanism. Our recent results (J. Hendriks, L. Whitehead and D. Day, unpublished data) indicate that the *B. japonicum* bacteroid carrier catalyses a $2H^+$/malate^{2-} symport, utilising the ΔpH across the bacteroid membrane. The PBM carrier, on the other hand, apparently catalyses electrophoretic transport of the univalent malate anion (Udvardi et al., 1988a), and utililises the $\Delta\psi$ across the membrane. These transport mechanisms are illustrated in Fig. 2.

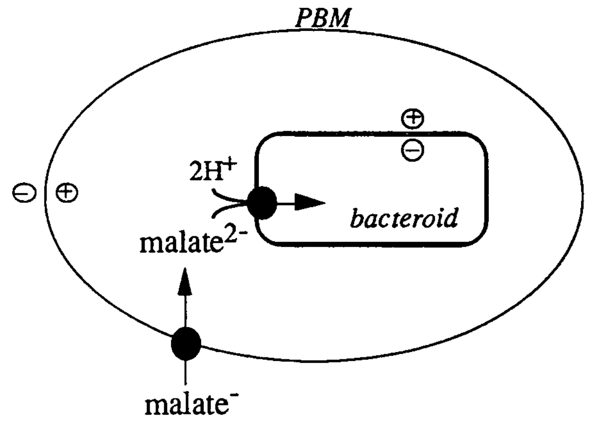

Figure 2. Malate transport across the PBM and bacteroid membranes:membrane energisation as in Fig. 1.

Other possible carbon sources

Although dicarboxylates are probably the major source of reduced carbon supplied to the bacteroid, in some symbioses other compounds may supplement this. For example, french bean symbiosomes take up glucose (but not other sugars: Herrada et al., 1989). Most legume nodules contain large quantities of malonate (Steeter 1991) and a case has been put for this organic acid as a carbon substrate (Kim and Chae, 1990). However, the PBM dicarboxylate carrier of soybean has a much greater preference for malate and at equimolar concentrations of the two acids, malonate transport would be severely restricted (OuYang et al., 1990). In soybean, siratro and french bean, glutamate and other amino acids are not likely to be significant sources of carbon (Udvardi et al., 1988b; Herrada et al., 1989; OuYang and Day, 1992).

Nitrogen transport to the plant

Amino acids

Although the role of dicarboxylates as carbon source seems to be generally accepted, the form in which fixed nitrogen is supplied to the plant remains uncertain (Long and Staskawicz, 1993). Strong cases have been made for hypotheses which link nitrogen efflux to carbon supply via obligatory exchanges across symbiotic membranes. These hypotheses include: a malate/aspartate shuttle, in which malate and glutamate enter and 2-oxoglutarate and aspartate leave the bacteroid (Kahn et al., 1985); a proline shuttle linking supply of reductant to the bacteroid with purine synthesis in the plant (Kohl et al., 1988); and a malonamate shuttle (Kim and Chae, 1990). The malate/aspartate shuttle appears unlikely to operate in view of the impermeability of the PBM to glutamate in soybean, french bean and siratro (see above). Likewise, no transport mechanism for proline has been identified on the PBM of soybean (Udvardi et al., 1990). Malonamate transport across the PBM has not been measured directly to our knowledge, but malonate uptake, which is required in this scheme (Kim and Chae, 1990), is likely to be very slow in the presence of malate, as discused above. We therefore consider all of these schemes to be unlikely mechanisms for the major flux of fixed nitrogen to the plant.

Exchange of an amino acid for malate does not necessarily require operation of a complicated shuttle. For example, it has been reported (Kouchi et al., 1991) that both alanine and aspartate, which can be readily formed in the bacteroid from malate (McDermott et al 1989), efflux rapidly from isolated soybean bacteroids, and some efflux of these amino acids has been seen also in long term experiments with isolated symbiosomes (Rosendahl et al., 1992). We have re-investigated the transport of these amino acids in soybean symbiosomes and bacteroids. We have found that the latter have an active transport mechanism for the uptake of aspartate and, when the bacteroids are pre-loaded with radioactive aspartate, exchange for external malate occurs; however, no such transport of aspartate occurs across the PBM (L. Whitehead, S. Young and D.A.Day, unpublished results). Likewise, we have been unsuccessful in identifying a mechanism for alanine transport across the PBM. It should be noted that some amino acids diffuse across the PBM at measureable rates (OuYang et al, 1990) and this, coupled with the active transporters on the bacteroid membrane, may allow some "scavenging" of amino acids as supplementary nitrogen sources for the bacteroid.

Ammonium transport

The direct product of nitrogen fixation in the bacteroid is ammonia which will diffuse rapidly into the PBS, driven by its concentration gradient and the ΔpH across the bacteroid membrane (Fig.1). The acidic pH in the PBS will ensure that the bulk of ammonia is converted to the ammonium ion. Repression of the bacteroid ammonium carrier (Brown and Dilworth, 1975; Howitt et al., 1985), prevents re-uptake of NH_4^+ which is therefore available to the plant. However, a transporter is required on the PBM for the rapid movement of this ion. Previous studies, using the ammonium analogue, methylammonium, failed to find any evidence for a carrier (Udvardi and Day, 1990) but this technique has limitations, especially when looking at efflux rather than uptake.

Given the lack of amino acid transporters on the PBM, we have reinvestigated the movement of NH_4^+ in isolated symbiosomes using the patch clamp technique (Tyerman et al., 1995). The relatively large size of symbiosomes from mature soybean nodules (2-5 µm in diameter) allows gigaohm seals to be formed with small pipettes (0.3-0.8 µM tips).

It is very difficult to maintain the symbiosomes attached to the pipette without rupture (monitored by microscopy) and therefore all measurments have been performed with detached patches of an inside out configuration (i.e., the side of the PBM usually facing the PBS faced the external medium). In all such patches, time-dependent currents (from the PBS face to the cytosolic face) were recorded when either K^+ or NH_4^+ were present in the external medium and when the membrane potential was made more negative (cytosolic side with respect to the PBS side). In other words, when an electrical potential was induced across the membrane, similar to that which would be formed by the PBM ATPase, NH_4^+ ions flowed from the external medium into the pipette: this is equivalent to NH_4^+ flowing out of the symbisome. The kinetics of current activation and deactivation indicated that a voltage gated channel was responsible for the currents (Tyerman et al., 1995). These currents were rectified, with no movement occurring when the cations were placed on the pipette (cytosolic) side of the patch. *In vivo*, this would ensure that no movement of NH_4^+ back into the symbiosome would occur.

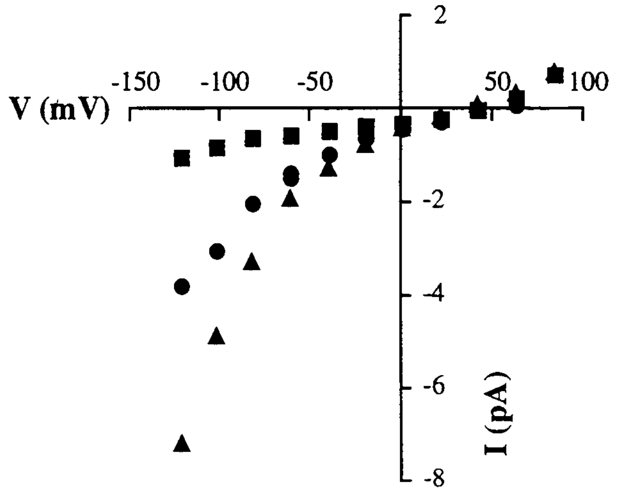

Figure 3. Patch-clamping of the peribacteroid membrane of isolated soybean symbiosomes. Current voltage curves of time-dependent current across inside-out patches of PBM. 30 mM chloride salts of choline (■), potassium (▲) and ammonium (●) were used in the external medium. The membrane voltage is cytoplasmic with respect to the peribacteroid space so that the current flows effectively out of the symbiosome.

Typical current reponses of a PBM patch to changing membrane voltage are shown in Fig. 3. The channel is selective for small monovalent cations, with K^+ and NH_4^+ being the most active, and does not transport divalent cations, larger cations such as choline (Fig. 3), or anions. In some patches, the conductance of the channel showed saturation kinetics with an apparent Km of about 30 mM for NH_4^+. This is close to the estimated concentration of ammonium inside symbisomes during nitrogen fixation (Streeter, 1989). The channel was strongly inhibited by calcium ions in the external medium, with a Ki of about 20 μM, perhaps indicating a potential regulatory mechanism for controlling NH_4^+ flux. The conductance of this channel is extremely small (subpicosiemen in amplitude) and discrete channel opening could not be detected readily. Non-stationary noise analysis of the currents recorded indicate that the channel is present in the PBM at very high density - 800-1000 channels per μm^2 (Tyerman et al., 1995) - consistent with the time-dependent current flows observed.

Although the channel described above has properties that one would expect of a transporter delivering ammonium to the plant, its operation during nitrogen fixation remains to be demonstrated. Experiments with symbiosomes isolated under micro-aerobic conditions should confirm this. Certainly the magnitude of the current flows across the patch, extrapolated to the whole symbisome, are quite adequate to account for estimates of nitrogen fixation rates in symbiosomes *in vivo* (Bergersen 1994).

A model for carbon/nitrogen exchange across the peribacteroid membrane

Based on the evidence reviewed here, the simplest scheme of carbon and nitrogen exchange between bacteroid and plant is malate and N_2 into the bacteroid for CO_2 and NH_4^+ out to the plant (Fig.4). The mechanisms required for this exchange are all present on the PBM of soybean nodules. Dicarboxylate uptake occurs via two carriers, one on the PBM which catalyses electrophoretic movement into the PBS and a proton symporter on the bacteroid membrane for uptake into the bacteroid. Malate (or succinate) can be completely oxidised in the bacteroid via malic enzyme and associated enzymes (Day and Copeland, 1991; see Finan, this volume). Ammonia formed in the bacteroid by nitrogenase will diffuse into the PBS where it will be converted to NH_4^+ which then enters the plant cytosol via the ammonium channel on the PBM. This exchange is coordinated via the membrane potentials of the PBM and bacteroid membrane, and the associated pH gradients (Fig. 1). Regulation of the exchange may occur via ATP concentration in the plant cytosol, which acts as a substrate for the ATPase and the protein kinase of the PBM, and the concentration of calcium on both sides of the PBM. Calcium is a potential regulator of carbon/nitrogen exchange across the PBM: in the plant cytosol it is required for activity of the PBM protein kinase (Weaver et al, 1991) while Ca in the PBS inhibits the activity of the ammonium channel (Tyerman et al., 1995). Since bacteroids appear to possess rather large quantities of Ca, it is possible that release of this cation by the microsymbiont may control both carbon input and nitrogen delivery.

The ammonium channel that we have discovered on the PBM is different from any other cation channel so far identified on plant membranes, including the inwardly rectified potassium channel on the plasma membrane (Schroeder et al, 1994), which also transports ammonium ions, and the high affinity ammonium transporter from *Arabidopsis* (Ninnemann et al., 1994). The high density of the channel in the PBM may have some advantage for the regulation of ammonium flux to the plant: for a small particle like the symbisome, a large number of low conductance channels would allow finer control of fluxes both in the long term, via protein synthesis, and in the short term via the percentage of channels in the open configuration. It is also interesting to note that ammonium regulates the expression of plant glutamine synthase (Miao et al., 1991). It is possible that expression of other plant genes are controlled in this fashion and NH_4^+ flux through the

PBM channel may, therefore, be involved in the regulation of symbiotic exchange in more than one way.

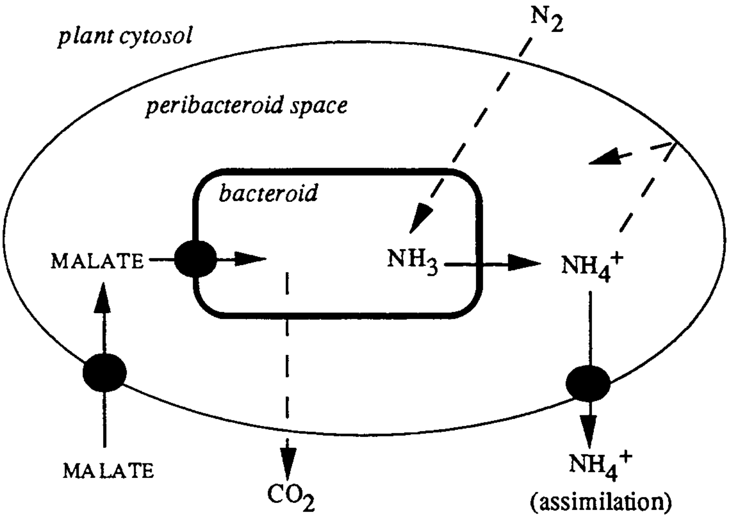

Figure 3. Simplest scheme for carbon/nitrogen exchange across the peribacteroid membrane of soybean nodules. Dashed lines represent diffusion.

Acknowledgements

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MALATE, ASPARTATE AND PROTON EXCHANGE BETWEEN *RHIZOBIUM LEGUMINOSARUM* SYMBIOSOMES AND ITS SYMBIOTIC PARTNER *PISUM SATIVUM.*

HAAKER, H., SZAFRAN, M.M. (1), WASSINK, H.J.
AND APPELS M.A.
Department of Biochemistry, Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands.
(1) Faculty of Commodity Science, Academy of Economics, al. Niepodleglosci 10, 60-697 Poznan, Poland.

INTRODUCTION

Carbon metabolism in the root nodule must fulfil two major tasks. The bacteroids must be supplied with oxidizable substrates and newly fixed nitrogen must be assimilated and transported to other parts of the plant. It is generally accepted that C_4-dicarboxylic acids are the major carbon source for the bacteroids to support nitrogen fixation, which are formed by the plant from sucrose (Boland et al. 1980, Werner 1992). The key reaction is the carboxylation of phosphoenolpyruvate catalyzed by phosphoenolpyruvate carboxylase (EC 4.1.1.31) (Christeller et al. 1977). The formed oxaloacetate is reduced by NADH to malate catalyzed by malate dehydrogenase (MDH).

Ammonia formed by the action of nitrogenase is excreted by the bacteroids into the host-cell cytoplasm. In the cytoplasm of the plant cell, ammonia is assimilated in glutamate by the action of glutamine synthase. The formed glutamine is metabolized further and depending on the type of plant, the newly fixed N is transported as an amide or ureide by way of the xylem of the host plant (Werner, 1992). For the assimilation of two molecules of NH_3, one molecule of aspartate is needed (Boland et al. 1980). It is assumed that aspartate is formed from oxaloacetate by the action of glutamate oxaloacetate transaminase (GOT) (Boland et al., 1980, Werner, 1992).

All experimental evidence indicates the activity of MDH and GOT determines the flux and distribution of oxaloacetate towards catabolism (malate formation which is oxidized by the bacteroids) and anabolism (aspartate synthesis for nitrogen assimilation). The activities and the kinetic constants of both enzymes present in the cytoplasm of pea nodule cells have been determined (Appels and Haaker, 1988 and 1991) but the relation between both enzyme activities, nitrogen fixation and ammonia assimilation was not quantified. In this paper the flux of oxaloacetate to malate catalyzed by MDH and to aspartate catalyzed by GOT was accessed.

An other enzyme present in nodules of which its role in nitrogen fixation is not clear, is the ATPase present in the peribacteroid membrane. In the root nodules of legumes this plant-derived membrane separates the nitrogen fixing bacteroids from the cytoplasm of the plant cell. This membrane contains an ATPase. To our knowledge no information is available about the effects of an active peribacteroid membrane ATPase on the nitrogenase activity of the symbiosomes (peribacteroid membrane enclosed

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bacteroids). In this report the electrogenic properties of the ATPase of peribacteroid membrane vesicles from pea nodules will be presented. Experimental data will be presented indicating that the ATPase activity, via the pH of the peribacteroid space, and dependent on the pH of the medium, stimulates or inhibits nitrogenase activity of the enclosed bacteroids.

Rate of aspartate formation in nodule cells

With isolated bacteroids the stoichiometry of malate and O_2 consumption and nitrogenase activity have been determined. When the nitrogenase activity is not inhibited by excess O_2 per mole malate consumed about 3 mol O_2 (2.8 ± 0.3) were used and 1.4 ± 0.2 mol C_2H_4 was formed. It was calculated from the reaction equation for the complete oxidation of malate ($C_4H_6O_5 + 3O_2$ ® $4CO_2 + 3H_2O$), that per mole C_2H_4 formed 2.7 mol of CO_2 are produced. This value agrees nicely with the respiratory cost of the acetylene reduction of 2.8 determined for detached pea nodules (Witty et al. 1983). They also showed that the respiratory cost of the acetylene reduction did not change between O_2 concentrations from 10% to 70%. We found for detached nodules that within the pO_2 range of 0.05 to 0.4 atm, the electron allocation to N_2 was constant (69 ± 9 %). Based on these data, it is possible to estimate from the C_2H_2 reduction rate, the minimal flux of oxaloacetate towards malate (to be oxidized by the bacteroids) and towards aspartate (used for the assimilation of NH_3).

The maximal rate of C_2H_2 reduction of detached nodules at a pO_2 of 0.4 atm is 220±18 nmol C_2H_2 reduced/min/g fresh nodule mass. This activity is equivalent to 220*0.69*2/3=101 nmol NH_3 (NH_3 takes up three reducing equivalent and C_2H_4 two). To assimilate 101 nmol of NH_3, 51 nmol oxaloacetate is necessary. With a minimal ratio of 2 for O_2 consumed / C_2H_2 reduced, 220*2/3=147 nmol malate will be oxidized to support nitrogenase activity.

Phoshoenolpyruvate carboxylase, MDH and GOT are supposed to be involved in the oxaloacetate metabolism in the cytoplasm of pea nodule cells. During nodule formation the specific activity of these three enzymes increases. The specific activity of phoshoenolpyruvate carboxylase increases 15 fold to 0.3 µmol/min/mg protein, MDH increases 2.5 fold to 25 µmol malate/min/mg protein) and GOT 2 fold to 2.9 µmol aspartate/min/mg protein. It was assumed that the increased activities of MDH and GOT are able to handle the necessary fluxes towards malate and aspartate respectively. The isolation procedure used to prepare the different nodule fractions resulted in a cytoplasmic plant fraction of 3.5 mg cytoplasmic protein/ml/g fresh weight nodule. This value was used to compare the calculated rates of malate and aspartate formation from oxaloacetate in nodules to support nitrogen fixation and assimilation with the measured MDH and GOT activities in the cytoplasmic plant fraction. As calculated above at least 42 nmol malate (oxidized by the bacteroids) must be formed/min/mg cytoplasmic protein to support the nitrogenase activity of the bacteroids and 15 nmol aspartate/min/mg cytoplasmic protein is needed for the assimilation of NH_3 (the synthesis of asparagine). The activities of the nodule stimulated forms of MDH and GOT under v_{max} conditions are 12.2 µmol malate and 3.0 mmol aspartate formed per min per mg cytoplasmic protein respectively. In principle these activities are high enough to support malate production for nitrogen fixation (44 nmol malate/min/mg protein) and aspartate formation for the NH_3 assimilation (15 nmol aspartate/min/mg protein). It must be realized that the enzyme activities have been measured under saturating substrate concentrations. To calculate the activity under non-saturating substrate and product concentrations the reaction mechanism and the kinetic constants are necessary. The MDH and GOT activities in the cytoplasmic plant fraction have been analyzed (Appels and Haaker, 1988, 1991). MDH could be separated into four fractions and GOT in three fractions. The nodule stimulates forms contain more than 70% of the total activity. The kinetic constants of the nodule dominant fractions are given in Table I. The kinetic data were in agrement with an Ordered Bi Bi mechanism for MDH and with a Ping Pong Bi Bi mechanism for GOT. The rate equations as derived by Cleland (1963) have been used in the calculations. The activities of the enzymes have been calculated with the following substrate concentrations: 0.02 mM oxaloacetate, 2.9 mM glutamate, 0.02 mM 2-oxoglutarate, 3.2 mM aspartate, 0.1 mM NADH, 0.5 mM NAD^+ and 5.0 mM malate (Appels and Haaker, 1991).

Table I The kinetic constants for MDH fraction IV and GOT fraction II.

kinetic constant[a]			cytoplasmic root nodule fraction	
	MDH	GOT	MDH fraction IV	GOT fraction II
K_a	(NADH)	(glutamate)	0.013	27
K_b	(oxaloacetate)	(oxaloacetate)	0.018	0.14
K_p	(malate)	(2-oxoglutarate)	2.6	0.23
K_q	(NAD^+)	(aspartate)	0.027	4.9
K_{ia}	(NADH)	(glutamate)	0.18	24
K_{ib}	(oxaloacetate)	(oxaloacetate)	0.025	0.062
K_{ip}	(malate)	(2-oxoglutarate)	3.5	0.60
K_{iq}	(NAD^+)	(aspartate)	3.8	6.4
K_{eq}			$61*10^3$	2.42
V_1/V_2[b]			20	2.85
V_1			12.2	2.99
V_2			0.61	1.05

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[a] The K_m and K_i-values are in mM; V_1 and V_2 are expressed in µmol.min^{-1}.(mg cytoplasmic protein)$^{-1}$. [b]V_1 is the maximum velocity for the formation of malate and NAD^+ (MDH) or aspartate and 2-oxoglutarate (GOT); V_2 is the maximum velocity in the reverse reaction.

The calculation gives a rate of formation of 2.0 µmol malate/min/mg protein catalyzed by MDH and 0.29 nmol aspartate/min/mg protein catalyzed by GOT. The calculated MDH activity is high enough to replenish malate consumed by the bacteroids for oxidation. But the rate of aspartate formation is much to low to support aspartate formation necessary for the NH_3 assimilation. The reason for the low activity of GOT at physiological substrate concentrations is the relative high Km values for the substrates of GOT (see Table I). To obtain a rate of 15 nmol aspartate/min/mg protein, the oxaloacetate and glutamate concentrations must be increased. For instance with [OAA] = 0.63 mM or [glu] = 85 mM or [OAA] = 0.1 mM and [glu] = 17 mM the calculated rate is 15 nmol aspartate/min/mg protein. But all mentioned concentrations are physiologically not relevant for the cytoplasm of nodule cells. Since PEP carboxylase and MDH are both located in the same cell compartment and due to the high MDH activity compared with the PEP carboxylase activity, it is expected that the oxaloacetate concentration will always be low (<20µM). Our calculation is in agrement with the observation of Snapp and Vance (1986) that GOT functions in the intact legume nodule in the formation of oxaloacetate from aspartate.

Since our calculations indicate that GOT present in the cytoplasm of the nodule cells probably is not able to catalyze the formation of aspartate at a significant rate, other possibilities to produce aspartate must be considered. This will probably take place in an other cell compartment. Possibilities are the malate/aspartate shuttle present in the mitochondria or the similar type of reactions catalyzed by the bacteroids (Appels and Haaker, 1991). It has been shown that malate and glutamate outside the bacteroids are converted into aspartate and 2-oxoglutarate. In a typical experiment at physiological substrate concentrations, 5 mM malate, 5 mM glutamate, 2.1±0.2 nmol aspartate/min/mg protein was secreted from the bacteroids. This rate was found to be independent of the rate of malate oxidation and nitrogenase activity. Thus at low nitrogenase activity enough aspartate is produced for the assimilation of the NH_3 formed but at the maximal nitrogenase activity (18.5±3.2 nmol C_2H_4 formed/min/mg protein) about 50% of the aspartate needed for the assimilation of NH_3 can be synthesized by the bacteroids. We like to conclude from our results that the activity of GOT in the cytoplasmic plant fraction is not high enough the provide the cell enough aspartate for the NH_3 assimilation. A possible role for the mitochondria and bacteroids is suggested.

Energization of peribacteroid membrane vesicles

Bacteroids within the infected cells of a legume are enclosed by a plant-encoded membrane, the peribacteroid membrane. This membrane is a possible site of transport regulation. It has also been reported that isolated peribacteroid membrane contains an ATPase. We studied the effect of an active peribacteroid membrane ATPase on the nitrogenase activity of peribacteroid membrane enclosed bacteroids (symbiosomes).

Peribacteroid membrane vesicles from pea nodules were isolated from symbiosomes. The ATPase activity associated with this membrane preparation was characterized and its electrogenic properties were determined. The apparent K_m value for MgATP was 0.52 ± 0.06 mM with a V_{max} of 1250 ± 60 nmol ADP.mg^{-1} protein.min^{-1} at 30 °C. The inhibition constant (K_i) for vanadate, which competitively inhibited the ATPase activity was determined from Lineweaver-Burk plots and was 58 ± 4 µM.

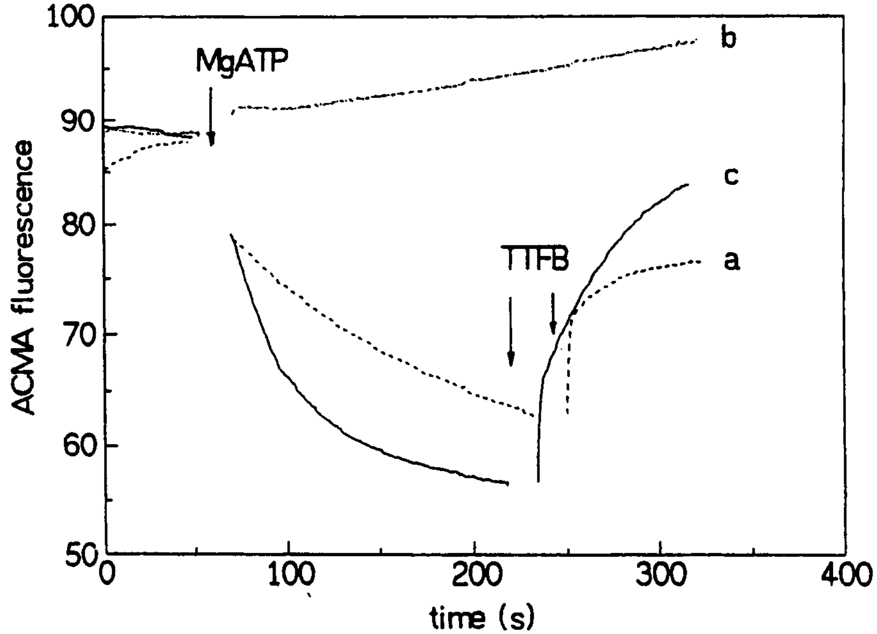

Figure 1 Changes of ACMA fluorescence upon addition of MgATP to symbiosomes and isolated peribacteroid membranes: (a) symbiosomes, (b) osmotically shocked symbiosomes and (c) peribacteroid membranes. The medium contained 50 mM Tes/KOH (pH 7.4), 5 mM $MgSO_4$, 50 mM K_2SO_4, 5 mM KCN, 1 μM ACMA, 250 μg bacteroid or 20 μg peribacteroid membrane protein.

The ΔpH was measured as a change of the fluorescence intensity of 9-amino-6-chloro-2-methoxy acridine (ACMA) and the membrane potential as a shift of absorbance of bis-(3-propyl-5-oxoisoxazol-4-yl)pentamethine oxonol. In Fig. 1 the effect of MgATP on the ACMA fluorescence is shown. As can be seen the ATPase generates a pH gradient across the peribacteroid membrane of symbiosomes (curve a) or isolated peribacteroid membrane vesicles (curve c). No effect was found with symbiosomes of which the peribacteroid membrane was removed by an osmotic shock (curve b). The observed quenching of the fluorescence of ACMA was reversed by the protonophore TTFB. It was also possible to reverse the fluorescence quenching by the addition of the inhibitor of the ATPase activity vanadate and by NH_4Cl (not shown). NH_4^+ dissipates a ΔpH by diffusion as NH_3 through membranes and reacting inside with H^+. Nitrate increased the fluorescence quenching further. Nitrate dissipates a membrane potential by moving into the vesicles in parallel with H^+ pumping. In the absence of transport systems NO_3^- collapses the ΔΨ and increases the ΔpH and this was observed for peribacteroid membrane vesicles.

For soybean symbiosomes the generation of a membrane potential (positive inside) across the peribacteroid membrane was demonstrated (Udvardi and Day, 1989; Udvardi et al., 1991). We were able to confirm these results with isolated peribacteroid membrane vesicles (not shown). The addition of MgATP caused a red shift of the absorbance of Oxonol VI at 630 nm relative to 608 nm which is indicative of the formation of a membrane potential. The addition of valinomycin, the protonophore TTFB and NO_3^-, all collapsing the membrane potential, reversed the spectral shift. The inhibition of ATPase activity by the addition of vanadate (0.4 mM) had the same effect. NH_4Cl (10-50 mM) did not collapse the Oxonol VI response. This indicates that NH_4^+ only dissipates the ΔpH but not the ΔΨ. In soybean the ΔΨ is lowered by Cl^-, indicative for a significant permeability in relation to the ATPase activity (Udvardi and Day, 1989). This is not the case for pea peribacteroid membrane vesicles.

Reversibility of peribacteroid membrane ATPase

In principle the P-type ion-motive ATPases are reversible. The slight stimulation (20%) of the ATPase activity by the addition of protonophores to sealed vesicles indicates inhibition by the proton motive force. To prove the coupling between ATP hydrolysis and H^+ pumping directly the reversibility of the peribacteroid membrane ATPase was tested. Peribacteroid membrane vesicles were fused with bacteriorhodopsin-phospholipid vesicles. As shown in Table II it was possible to demonstrate a protonophore-sensitive light-dependent ATP synthesis. The light dependence and the protonophore-sensitive ATP synthesis excludes the possibility that ATP is formed due to adenylate kinase activity. To exclude the probability that a minor contamination of the peribacteroid membrane preparations with cytoplasmic membranes derived from bacteroids causes the ATP synthesis via its F_o-F_1-ATPase, cytoplasmic membrane vesicles isolated from bacteroids were also used in a reconstitution experiment. When cytoplasmic membranes vesicles were used in an amount corresponding to the maximal contamination observed in peribacteroid membrane preparations (15% on protein basis), no light-dependent ATP formation was observed (not shown).

Table II The light-dependent ATP synthesis of purple membrane-phospholipid vesicles (BR-PC) and peribacteroid membrane-purple membrane-phospholipid vesicles (PM+BR-PC).

Treatment	BR-PC	PM+BR-PC
	ATP formation ($nmol.min^{-1}.mg^{-1}$)	
3 min hυ	0	28.9
6 min hυ	0	26.7
6 min hυ + 2 μM TTFB	0	0

Effect of energization of the peribacteroid membrane on the nitrogenase activity of bacteroids

The effect of the peribacteroid membrane ATPase activity on respiration and nitrogen fixation of symbiosomes has been studied. The pH dependence of the nitrogenase activity of symbiosomes oxidizing malate shows a broad optimum between pH 6.5 and 7.4. There is no activity at pH 4.0 and 8.6. The effect of the energization of the peribacteroid membrane on the nitrogenase activity was determined at pH 6.5, 7.4 and 8.4. The results are presented in Table III. At pH 7.4 and pH 6.5 (data not shown) the addition of MgATP to the symbiosomes strongly inhibited nitrogenase activity.

Inhibition of the peribacteroid membrane ATPase by vanadate or the dissipation of the pH gradient by NH_4Cl reversed the inhibition by MgATP. O_2 titration experiments demonstrated that the optimum pO_2 for nitrogenase activity did not change significantly. It can therefore be excluded that the inhibition induced by MgATP is caused by O_2 inhibition of nitrogenase. At pH 8.4 where the pH of the medium inhibited the nitrogenase activity, the activity of the peribacteroid ATPase did not inhibit but stimulated the nitrogenase activity slightly.

Table III Effect of pH and energization of the peribacteroid membrane on the nitrogenase activity of *R. leguminosarum* symbiosomes

Additions	% of the maximal nitrogenase activity[a] pH 7.4	% of the maximal nitrogenase activity[a] pH 8.4
-	100 ±4	100 ±4
MgATP	39 ±6	120 ±5
MgATP + NH_4Cl	80 ±4	
MgATP + Na_3VO_4	85 ±4	102 ±6
NH_4Cl	94 ±3	
Na_3VO_4	84 ±3	97 ±6

[a] 100% activity at pH 7.4 and 8.4 is 17.3 and 6.1 nmol C_2H_4 formed. min^{-1}. mg^{-1} protein respectively. Each value represents the mean of three replicates ± 1 SE

Table IV Effect of vanadate on nodular nitrogenase activity

Additions	% of nodular nitrogenase activity
15 mM NaCl	100±15
15 mM Na_3VO_4	127±16

100% activity is 32 nmol C_2H_4 formed.min^{-1}.g^{-1} fresh weight. Each value represents the mean of six replicates ± 1 SE.When the peribacteroid membrane ATPase is also active in intact root nodules, nitrogenase activity might be partially inhibited. This hypothesis was tested. The air spaces in the root nodules were filled with a vanadate solution by evacuating the root nodules in this solution. The loss in nodular nitrogenase activity due to submerging was relieved by increasing the pO_2 to 80% v/v. The effect of vanadate was compared with a control experiment with NaCl. As shown in Table IV vanadate stimulates nodular nitrogenase activity. We like to explain this stimulation as an inhibition of the peribacteroid membrane ATPase and thus by increasing the pH of the peribacteroid space to a more optimal pH for nitrogen fixation by the bacteroids. It is realized that vanadate will also inhibit other ATPases like the plasma membrane H^+-ATPase. Further work must clarify the precise mechanism of action of the vanadate stimulation of nodular nitrogenase and must find out if the peribacteroid membrane ATPase activity can be regulated as described for the plasma membrane H^+-ATPase (Serrano, 1989).

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Footnotes

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MOLECULAR GENETICS OF C_4-DICARBOXYLIC ACID TRANSPORT IN *RHIZOBIUM TROPICI.*

BATISTA S.(1), HERNANDEZ-LUCAS I.(2),
MARTINEZ-ROMERO E.(2), MARTINEZ-DRETS G.(1).
(1) Biochemistry Division, IIBCE, Ave.Italia 3318, 11600, Montevideo, Uruguay, (2) Departamento de Genetica Molecular, CIFN, UNAM, 565-A, Cuernavaca, Mexico.

The symbiotic association *Rhizobium*-legume involves the exchange of both signals and metabolic substrates between the two partners. Previous studies demonstrated that C_4-dicarboxilic acids (DCA) are essential substrates for the bacteroid in some species of *Rhizobium*. The objective of this work is to determine the role of DCA transport system in *R.tropici*. A *dct*-like region from *R.tropici* CIAT899 was cloned in pSUP205. This clone was isolated from a genomic bank using *dctABD* from *R.meliloti* as a probe. From this clone two fragments EcoRI of 6.40kb and 4.50kb hybridized with *R.meliloti dctABD* but only the 4.5-kb fragment hybridized with 955 pb internal region of *R.meliloti dctA*. These fragments were subcloned in pRG960sd and transferred to *R.meliloti dct* mutants in order to test their abilities to complement their mutations. RmF642 (*dctA*::Tn5) carrying the 4,5-kb fragment in pRG960sd and RmF121 (*dctD*::Tn5) with the 6.4-kb fragment in the same cosmid could grow on minimal medium with succinate as carbon source suggesting that these plasmids complemented their mutations.In order to localize the *dct*-like operons, megaplasmid profiles of *R.tropici* CIAT899, CFN299, *R.elti* CE3 and BR10030 were hybridized with *R.meliloti dctABD* as probe. The results showed that these genes are located on a plasmid and agree with the previous results obtained with *R.meliloti*.

Subsequently the 4.5-kb fragment EcoRI was cloned in the sucrose sensitive plasmid pWS233. This plasmid is being used to isolate mutants of *dct*-like genes by insertion of Tn5mob. These mutants will be used to determine the importance of *dct* system in *R.tropici* during the symbiotic nitrogen fixation in *Phaseolus vulgaris*.

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ORGANIZATION OF THE PHB SYNTHASE GENE LOCUS AND ITS GENETIC REGULATION IN *RHIZOBIUM ETLI*

CEVALLOS, M.A., MARTINEZ A., LEIJA, A., ENCARNACION, S., AND MORA, J.
Centro de Investigacione Sobre Fijacion de Nitrogeno, UNAM.
Av.Universidad s/n, AP-565-A Cuernavaca, Mor., MEXICO.

R. etli accumulates large amounts of Poly-beta-hydroxybutyrate (PHB) in free life and in symbiosis. But species like R. meliloti accumulates PHB in free life and in the infection threads but not in nitrogen fixing bacteroids. In free life PHB accumulation is part of a pseudofermentative metabolism that is also characterized by the excretion of organic acids and amino acids; by an increased cellular aggregation and by the reduction of growth rates by a 50% during serial subcultivations in minimal medium. A Pseudofermentative response can be induced in several ways, for example, by inoculating the medium at high cell density or by growing the cells in a minimal medium with pyruvate as carbon source and nitrate as nitrogen source. An aerobic metabolism can be maintained if the minimal medium is inoculated at low cell density or if the medium is supplemented with biotin or thiamine. In order to study the genetic regulation of PHB synthesis we constructed a transcriptional GUS fusion within the PHB synthase gene (phaC) of R. etli to determine its expression in symbiosis and in free living state under conditions favoring or suppressing PHB accumulation. This fusion was also, introduced in R. meliloti to analyze its expression in symbiosis and in free life.

Studies of the phaC-GUS fusion indicates that the phaC gene is expressed constitutively at the same level under conditions leading to PHB accumulation and under conditions where no PHB accumulates with a variation that not exceed two fold. However, the PHB concentration was at least 10 times higher. This result suggests that PHB accumulation is regulated by the rate of PHB depolymerization and/or by the amount of carbon which is channeled to PHB synthesis.

In symbiosis, nodules of bean plants induced by R. etli containing the phaC-GUS fusion expressed beta-glucuronidase in the infected cells in all stages of development. In contrast, nodules of alfalfa plants induced by R. meliloti containing the phaC-GUS fusion only expressed beta-glucuronidase in the early symbiotic region suggesting some kind of developmental regulation.

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POLY-B-HYDROXYBUTYRATE: WHY DOES IT ACCUMULATE IN SOME NITROGEN - FIXING LEGUME SYMBIOSES BUT NOT IN OTHERS?

COPELAND L. and KIM S.A.
Department of Agricultural Chemistry and Soil Science,
University of Sydney, NSW Australia 2006

Bacteroids in root nodules of numerous legume species contain substantial amounts of poly-ß-hydroxybutyrate (PHB), a polyester storage reserve which is considered to have an important role in nitrogen fixation by providing a readily oxidizable source of carbon and reductant when exogenous substrates are not available. However, some species of bacteroids do not accumulate PHB and the reason for this is not clear. In this study, we have compared bacteroids of soybean (*Glycine max* L.), which may contain large amounts of PHB, and chickpea (*Cicer arietinum* L.) which have been shown by electron microscopic examinations to contain little or no PHB.

Gas chromatographic analyses confirmed that soybean bacteroids and the corresponding free-living bacteria (*Bradyrhizobium japonicum* CB 1809) accumulated substantial amounts of PHB, as did cultures of the chickpea nodulating bacteria (*Rhizobium* sp. *Cicer* CC 1192). However, only traces of PHB were found in chickpea bacteroids.

Analysis of the capacity for PHB synthesis (3-ketothiolase and acetoacetyl-CoA reductase) and breakdown (PHB depolymerase and 3-hydroxybutyrate dehydrogenase), and related metabolic reactions (NAD- and NADP-malic enzymes, malate dehydrogenases, pyruvate dehydrogenase, citrate synthase, isocitrate dehydrogenase and glutamate:2-oxoglutarate aminotransferase), indicated that the potential activities of these enzymes were similar in extracts from soybean bacteroids and free-living *B. japonicum* CB 1809. Although enzymes of PHB synthesis were active in extracts from chickpea bacteroids, there were significant differences to the free-living bacteria. We suggest that these enzymic differences, and the environment within the nodules, may be important in determining whether PHB accumulates.

BIOCHEMICAL AND GENETIC CHARACTERIZATION OF PYRUVATE CARBOXYLASE IN *RHIZOBIUM ETLI* AND *RHIZOBIUM TROPICI*

DUNN, M. AND MORA, J.
Centro de Investigacion Sobre Fijacion de Nitrogeno, UNAM.
Av.Universidad s/n, AP-565-A Cuernavaca, Mor., MEXICO.

The biotin-dependent enzyme pyruvate carboxylase (PYC) catalyzes the conversion of pyruvate to oxaloacetate in eukaryotes and certain bacteria, and is crucial in allowing the TCA cycle to fulfill a biosynthetic as well as an energy-generating role. Physiological and biochemical studies in this laboratory (Encarnación, et al, J. Bacteriol. in press) have shown that PYC appears to play an important role in the aerobic metabolism of R.etli and R.tropici strains in vitro. Using transposon mutagenesis, we have inactivated the PYC gene in R.tropici CFN299 and, by complementation, have isolated, subcloned and partially sequenced the PYC gene from R.etli strain CE3.

The R.tropici PYC mutant, strain 2-176, was isolated based on its succinate$^+$ glucose$^-$ pyruvate$^-$ growth phenotype: the wild-type strain (CFN299) grows well on any of these carbon sources. Strain 2-176 lacks PYC activity, but produces normal amounts of other enzymes associated with oxaloacetate metabolism. Sequence analysis indicates that the Tn5 insertion lies within the biotin carboxylase domain of the PYC gene. The PYC mutant also grows well during serial subcultivation in a minimal medium containing succinate as a carbon source (MS), as does the parent strain. This finding indicates that PYC is not required for the growth of this strain on MS.

Sequence analysis of subcloned DNA from the PYC-complementing cosmid isolated from an R.etli strain CE3 genomic DNA library revealed a great degree of sequence homology with those of known PYC genes, and indicates that the gene encodes all three domains (biotin carboxylase, transcarboxylase and biotin carboxyl carrier protein)common to other PYC's.

Other interesting features of the PYC enzymes from strains CE3 and CFN299 are their near-absolute requirement for acetyl CoA, an activator of mammalian but not, normally, prokaryotic PYC's. The $K_{0.5}$ for acetyl CoA for the PYC from strain CFN299 is approximately 7 uM, and that of the strain CE3 PYC appears to be in a similar range. As expected for a biotin-containing enzyme, both PYC's are greatly inhibited by avidin, and this inhibition is prevented by the presence of excess biotin. The PYC produced by strain CE3 is inhibited 80 % in the presence of 5 mM L-aspartate, another feature common to mammalian, but not bacterial, PYC's (the PYC from strain CFN299 has yet to be tested for the effect of L-asp). Activation by acetyl CoA and inhibition by L-aspartate could play a regulatory role in vitro by controling the availability of oxaloacetate for catabolic and biosynthetic purposes. Gel permeation chromatography of the PYC isolated from CE3 indicates an apparent M_r of 400 KDa, wich is within the range reported for PYC isolated from other sources.

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ALTERNATIVE METABOLIC PROGRAMS IN *RHIZOBIUM*

ENCARNACION, S., DUNN M., LEIJA, A., VARGAS, M.C.,
PERALTA, H., AND MORA, J.
Departamento de Ecologia Molecular, Centro de Investigacion Sobre
Fijacion de Nitrogeno, UNAM. Av.Universidad s/n, AP-565-A
Cuernavaca, Mor., MEXICO.

The growth of *R. etli*, *R. tropici* and *E. meliloti* strains is impaired when subcultured from rich to minimal medium (MM) and this unbalanced growth is involved with a change from aerobic to fermentative metabolism. Each *Rhizobium* species has a specific pattern of diminishing growth on MM. Specific growth rates are directly related to oxygen consumption levels, poly-β-hydroxybutyrate (PHB) accumulation, the occurrence of cell aggregation and a drastic reduction in the activity of several enzymes of the TCA cycle. These phenomena have been extensively studied in *R. etli*, where in addition to PHB, other fermentative products such as oxoglutarate, malate, fumarate, glutamate, alanine, etc, are also produced and excreted. Some of these compounds are known chemoattractors. Coupled to this fermentative state, we found an increased oxidative damage resistance, relating the switch of the metabolic programs with cell aggregation. In support of this relation, addition of N-methyl-aspartic acid, an inhibitor of chemotaxis, prevents the change to the fermentative metabolism. In addition two of the key enzymes that supply substrates to the TCA cycle, pyruvate carboxylase (PYC) a biotin enzyme and pyruvate dehydrogenase (PDH) a thiamine enzyme, are very low during fermentation. If one of these vitamins are added to MM all these metabolic changes are prevented and an aerobic metabolism is established. We have found that the addition of biotin increases several-fold the activity of PYC and the addition of thiamine does the same for PDH activity. (Encarnación, S., Dunn, M., Willms, K., and Mora, J., J. Bacteriol. June 1995).

A transposon Tn-5 structural mutant lacking PYC has been selected from *R. etli*. This mutant does not grow in pyruvate or glucose but grows well in succinate. However, when this mutant is serially subcultured in MM containing succinate, it stops growing and develops a strong fermentative metabolism. It is clear that PYC supplies oxaloacetate (OA) through the carboxylation of pyruvate and that the lack of this enzyme favors the derivation of pyruvate for AcetylCoA formation and subsequent PHB synthesis, and a fermentative metabolism.

When the *R. etli* PYC$^-$ mutant was grown on succinate plus biotin, this vitamin had a diminished capacity to induce the aerobic metabolism and cells accumulated higher amounts of PHB in comparison with the wild-type strain. We conclude that in *R. etli* besides PYC, there is another carboxylating enzyme that requires biotin involved in the synthesis of OA. The carboxylation of pyruvate and the effect of biotin are central factors in sustaining an aerobic or fermentative metabolism in *R. etli*.

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THE RESPONSE OF ROOT NODULE METABOLISM TO DROUGHT IN SOYBEAN.

GORDON A.J.[1], GONZALEZ E.M.[2], ARRESSE-IGOR C.[2], JAMES C.L.[1].

1. Institute of Grassland & Environmental Research, Plas Gogerddan, Aberystwyth, Dyfed SY23 3EB. UK.
2. Dpto de Ciencias del Medio Natural, Universidad Pública de Navarra, Campus Arrosadía, E-31006, Pamplona, Spain.

Experiments were carried out to investigate the effects of drought stress on carbon and nitrogen metabolism in soybean nodules. Gradual mild, drought stress was imposed by withholding water/nutrients and resulted in declines in the water potential of leaves and nodules consistent with a 50-70% decline in N_2 fixation. However, the amounts of nitrogenase components 1 and 2 (assessed by Western blotting) were virtually insensitive to water stress. Similarly no significant changes could be detected in aspartate aminotransferase, phospho*enol*pyruvate carboxylase, glutamine synthetase or alkaline invertase activities or in the levels of leghaemoglobin through the 11 day experiment. In contrast, sucrose synthase, one of the enzymes involved in sucrose metabolism in legume nodules, declined dramatically in activity and in content within a few days of withholding water. Coincident with this decline in SS activity was an overall increase in carbohydrate content, the net result of a large increase in sucrose and a reduction in starch content. In addition there were significant increases in the amounts of total free amino acids and ureides although proline levels did not increase until some days later. It is suggested that SS may play a key role in the regulation of nodule carbon metabolism and, therefore, of nitrogen fixation under drought stress conditions.

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CARBON METABOLISM IN ALFALFA NODULES: MALATE DEHYDROGENASE.

GREGERSON, R.G.[1], DRISCOLL, B.T.[1], GANTT, J.S.[2],
VANCE, C.P.[1,3]
[1]Departments of Agronomy and Plant Genetics, [2]Plant Biology, and
[3]USDA ARS, The University of Minnesota, 411 Borlaug Hall, S.Paul,
MN 5508.

Organic acids derived from the degradation of photosynthate and from root nodule nonautotrophic CO_2 fixation are the primary source of energy for nitrogen fixation by symbiotic bacteroids. The plant enzyme malate dehydrogenase (MDH) is thought to play a critical role in the synthesis of organic acids in nodules. In order to more fully understand how organic acid metabolism is controlled in alfalfa nodules, we thought it important to characterize MDH gene expression. cDNAs for mitochondrial and glyoxysomal MDH were isolated from a nodule cDNA library, while a plastid MDH cDNA was isolated from a leaf cDNA library. The deduced amino acid sequ ences of the alfalfa glyoxysomal and mitochondrial MDHs are very similar to those previously reported from melon, and the chloroplast MDH is very similar to chloroplast MDHs from maize, sorghum, and ice plant. RNA blots show that the glyoxysomal and mitochondrial MDHs are expressed rather uniformly in all tissues. The plastid MDH shows preferential expression in green tissues. These three MDH forms do not show high level induction in root nodules as compared to other tissues. To isolate additional cDNA clones encoding malate dehydrogenase, we introduced an alfalfa nodule cDNA library, carried on plasmid pcDNA II, into *E. coli* Mdh$^-$ mutant 1477, which is unable to grow on minimal medium with succinate as sole carbon source. Two classes of plasmids complemented the succinate-growth phenotype of 1477. One class of MDH cDNAs encodes a putative cytosolic MDH, which appears to be expressed equally in all tissues. The second class of complementing cDNAs encodes an apparently unique MDH. While this MDH appears to be more closely related to mitochondrial and glyoxysomal forms, its transit peptide does not resemble mitochondrial or glyoxysomal targetting peptides. Preliminary Northern analysis indicated that this MDH is more highly expressed in nodule, than other alfalfa tissue. Analysis of Southern blots revealed that the nodule-enhanced MDH is encoded by a locus distinct from that of the previously characterized mitochondrial or glyoxysomal MDHs. Complementation tests revealed that plasmids carrying glyoxysomal, mitochondrial, cytosolic, or nodule-enhanced MDHs complemented the *E. coli* Mdh$^-$ mutant, but that plasmids carrying the chloroplast MDH did not.

POSSIBLE PARTICIPATION OF PROLINE IN THE OXYGEN DEFENSE SYSTEM IN LEGUME ROOT NODULES.

RADUKINA N.L. A.N. Bach Institute of Biochemistry, Leninsky prospect 33, Moscow, 117071, Russia.

Proline (Pro) accumulation is part of the plant adaptive system. As was shown, Pro is accumulated in the legume root nodules in the period of maximum nitrogen fixation. However, the Pro role in the legume root nodules is only little understood. Our experiments showed that the contents of free Pro were 26.5, 35.7, and 55.4 g/g fresh wt in the cortex zone of root nodules of *Glycine max, Vicia faba, Lupinus luteus,* respectively. The contents of bound Pro and hydroxy-Pro were considerable in the cortex of all the nodules investigated, presumably, due to the presence of various Pro-rich proteins in the cortex (extensine, early nodulins ENOD 5 and ENOD 12).

In the infected zone of investigated nodules the free Pro was detected both in the bacteroid and as in the plant cell cytosol. In the cytosol of ineffective lupin and bean nodules the free Pro content was doubled. Pro was absent in the bacteroid of both ineffective nodules. The activity of Pro DH increased and the activities of catalase and ascorbat peroxidase decreased in the ineffective cytosol. I propose that Pro may be involved in the pH and redox regulation in the cytosol of effective and ineffective root nodules. The higher Pro accumulation in the ineffective nodules is a symptom of metabolic disturbance induced by the decreased activity of oxygen detoxicating enzymes.

GLUTAMATE CYCLING MAINTAINS THE REDUCTANT FLOW FROM MALATE FOR NITROGEN FIXATION IN BACTEROIDS OF LEGUME ROOT NODULES: A HYPOTHESIS.

Shatilov V.R.
A.N.Bach Institute of Biochemistry, 33, Leninsky Prospect, Moscow, 117071, Russia.

Malate is generally considered to be the principal energy source for symbiotic N_2-fixing bacteroids (Streeter, 1991). I suggest that malate dehydrogenase (MDH) is a major enzyme for malate oxidation in bacteroids to provide N_2-fixation with energy. MDH is very active in bacteroids (Driscoll, Finann, 1993). As K_{eq} for MDH is very low ($\sim 10^{-5}$) (Stern et al., 1952) oxaloacetate formed on malate oxidation must be withdrawn to maintain continual malate oxidation. This is fulfilled by aspartate aminotransferase (AAT)((Kouchi et al., 1991). MDH and AAT function in a coupled manner as it takes place in Frankia vesicle cells (Vikman, Huss-Danell, 1991). The aspartate formed is splited to fumarate and ammonium by aspartase (Kouchi et al., 1991). The fumarate is transformed to malate by fumarase. It appears that the TCA cycle in N_2-fixing bacteroids operates in an abnormal way: the reactions from oxaloacetate to succinate run at a slow rate because of a very low activity of 2-oxoglutarate dehydrogenase and its strong inhibition by NADH (Salminen, Streeter, 1990), while the reactions from succinate to oxaloacetate run at a high rate. The inhibition of pyruvate dehydrogenase, citrate synthase and isocitrate dehydrogenase by NAD(P)H may contribute to this way of TCA functioning (see review Mc Dermott et al., 1989). If this is the case then the phenomenon when *Rhizobium leguminosarum* mutant lacking pyruvate dehydrogenase grows well on succinate and induces fix$^+$ nodules (Glenn et al., 1984) could be explained. The ammonia formed is reassimilated through the glutamine synthetase reaction. The glutamine is then transformed to glutamate by glutamate synthase. Thus the molecule of glutamate taken on malate oxidation is regenerated, and in this way the cycling of glutamate takes place to maintain the NADH flow from malate in N_2-fixing bacteroids.

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SUCROSE TRANSPORT AND METABOLISM IN *RHIZOBIUM TROPICI.*

SOF'IN A.V.(1), ROMANOV V.I.(1), TCHERMENSKAYA I.E.(1), MARTINEZ-ROMERO E.(2),
(1) A.N.Bach Institute of Biochemistry, Leninsky prospect 33, Moscow, 117071, Russia.
(2) CIFN-UNAM, Cuernavaca, Morelos, Mexico.

The process of sucrose utilization by Rhizobia could be important in the formation of effective nodules, but information concerning sucrose transport and metabolism in these bacteria is very limited.

Rhizobium tropici CFN 299 was maintained on PY medium and was grown in minimal medium with sucrose, glucose, fructose, malate and glutamate (or their combination) as carbon sources and nitrate or ammonia as nitrogen sources. Bacteria were able to use simultaneously different carbon compounds. Sucrose transport was active, induced by sucrose and partially repressed by glucose and glutamate. Nonlinear Lineweaver-Burk plots for the sucrose uptake (by sucrose-grown bacteria) implies the existence of at least two uptake mechanisms.

The activities of 22 enzymes involved in the carbon and nitrogen metabolism in free-living cells and in bacteroids, isolated in Percoll gradient were determined. The set of activities of the carbon metabolism enzymes suggests that PP-pathway, with participation of the ED-pathway is probably the primary route for sugar catabolism. In bacteroids the activity level of many enzymes measured were similar to those of malate-grown cells, except higher activities of glucokinase, glucose-6-phosphate dehydrogenase and NAD phosphogluconate dehydrogenase.

In sucrose-grown cells a nitrogen source (nitrate or ammonia) affected the activity levels of either nitrogen (NAD glutamate synthase and glutamine synthetase) or carbon metabolism enzymes (NAD 6-phosphogluconate dehydrogenase, NAD- and NADP-malic enzymes, and β-hydroxybutyrate dehydrogenase).

In conclusion, the results obtained support the idea that *Rhizobium tropici* is adapted to sucrose utilization and multicarbon nutrition during interaction with plants.

INVOLVEMENT OF CARBOHYDRATE METABOLISM ON THE REGULATION OF NITROGENASE ACTIVITY IN WHITE CLOVER NODULES.

WEISBACH, C., HARTWIG, U.A., HEIM, I. and NÖSBERGER, J.
Institute of Plant Sciences, Federal Institute of Technology,
8092 Zürich, Switzerland.

The fast and dramatic decrease in nitrogenase activity in legume nodules after plant defoliation is associated with an increased oxygen limitation of nitrogenase activity, presumably caused by a reduced oxygen permeability in nodules (1, 2). Carbohydrates from current photosynthesis or from reserves were disproved to have a direct metabolic function in the short term down-regulation of nitrogenase activity (3, 4). However, the possibility that compounds from the carbohydrate metabolism are indirectly involved in triggering the oxygen permeability through e.g. osmotic processes (5) was never tested. To test this hypothesis, nodule oxygen permeability was manipulated by either defoliation or with various rhizospheric pO_2. None of the treatments affected starch, fructose, L-malate, D-pinitol or succinate concentrations. Nodule sucrose concentration showed a decline due to defoliation while the reduced O_2 permeability induced by 40 kPa O_2 had no effect. These inconsistencies between nodule sucrose concentration and O_2 permeability upon various treatments do not support the hypothesis that whole nodule sucrose concentration triggers nitrogenase activity. The fact that the response of carbohydrate metabolites in roots was parallel to that in nodules would suggest that supply of carbohydrates to the nodules is regulated by the availability from the shoot. To test this hypothesis, sucrose concentration in the nodules was monitored after 40% leaf area removal, known to have no effect on nitrogenase activity (4). In this experiment, sucrose concentration decreased, suggesting that the supply to the nodules was significantly reduced. Obviously, the sucrose pool in nodules is always above a level to support required nitrogenase activity. However it can not be excluded that carbon compounds are affecting nitrogenase activity by shifting metabolites inbetween nodule compartments.

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PHOSPHO*ENOL*PYRUVATE CARBOXYLASE IN SOYBEAN NODULES

WADHAM C., WINTER H., SCHULLER K. A.
School of Biological Sciences, Flinders University, G.P.O. Box 2100,
Adelaide, S.A. 5001, Australia

Phospho*enol*pyruvate carboxylase (PEPC) is a key enzyme in the provision of respiratory substrates (mainly malate) by the plant to the N_2-fixing bacteria (bacteroids) in soybean nodules. We have isolated two different forms of the enzyme with different K_m(PEP) and K_i(malate) values. The form with the lower affinity for PEP and greater inhibition by malate is found in the nodules of plants which have had their shoots removed prior to sampling suggesting that the less active form of PEPC may arise under conditions of "photosynthate deprivation". Incubation of crude extracts of the less active form of the enzyme with ATP results in its conversion to the more active form. By the same token, incubation of the more active form of the enzyme with alkaline phosphatase results in its conversion to the less active form. These data indicate that the two different forms of the enzyme represent different phosphorylation states.

BIOCHEMICAL CHARACTERIZATION OF PLANT-CONTROLLED INEFFECTIVE NODULES OF PEA (Pisum sativum L.).

DOLGIKH E., FEDOROVA M., FILATOV A., TIKHONOVICH I.
All-Russia Research Institute for Agricultural Microbiology,
St-Petersburg-Pushkin 8, 189620, RUSSIA

Ineffective Fix$^-$-lines carrying mutations in three different sym-loci (Sprint2Fix$^-$, SGEFix$^-$-1, SGEFix$^-$-2) and their parental forms (Sprint2 and SGE) [1,2] were used to study the key enzymes of nodule N and C metabolism related to assimilation of symbiotically fixed nitrogen. Effective pea nodules possess several times higher NADH-GOGAT, PEPC, AAT and MDH specific activities than ineffective nodules or uninfected roots. The increased activities of enzymes are determined by the synthesis of nodule-enhanced isoforms, as it has been demonstrated for NADH-GOGAT, PEPC and AAT. Plant-controlled nodule inefficiency leads to similar changes in specific activities of enzymes mentioned before as well as in the content of nodule-enhanced isoforms disregarding the particular sym-mutation. The amount of each polypeptide in ineffective nodules is much lower (PEPC, AAT-2) or undetectable (NADH-GOGAT) comparatively with effective ones, as demonstrated by immunoblotting with monospecific antibodies.

Nodule enzyme activities begin to increase as soon as the effective nodules start to develop. A slight flash of the enzyme activities occurs at the same stage of development in ineffective nodules. Thus the existence of a general signal acting during nodule morphogenesis and controlling nodule-enhanced synthesis can be suggested. Nitrogen fixation is responsible for an additional signal that increases enzyme activities in the effective nodules; this signal is absent in ineffective nodules of the same age.

Ammonium ions appear not to induce the nodule-enhanced synthesis of NADH-GOGAT and AAT; other mechanisms regulating the enzyme expression might exist in pea nodules. The obtained data can be usefull for future study of the molecular basis of symbiosis-specific induction of genes controlling nodule metabolism.

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EFFECT OF DROUGHT STRESS ON PROLINE UTILIZATION IN SYMBIOSOMES FROM SOYBEAN (*GLYCINE MAX* L.) ROOT NODULES.

FELDNER H.C., PEDERSEN A.L. and ROSENDAHL L.
Environmental Science and Technology Department,
Risø National Laboratory, DK-4000 Roskilde, Denmark.

Introduction. Soybeans subjected to drought stress accumulate proline in the nodules and the bacteroids have an increased capacity to oxidize proline (Kohl *et al.*, 1991). There is a limited passage of proline across the peribacteroid membrane (PBM) in non-stressed soybeans (Udvardi *et al.*, 1990) and malate is believed to be the main substrate for the bacteroids. There has to be a change in the permeability of PBM to proline during periods of drought stress for the bacteroids to use proline as a source of energy.

The present work describes the effect of drought stress 1) on the ability of bacteroids to utilize proline to support nitrogenase activity and 2) on the uptake of proline by symbiosomes.

Materials and methods. Six-week-old soybeans were subject to 40 hours drought. This results in a 60% decrease in nitrogenase activity (acetylene reduction) which recovers upon rewatering. All handling of nodule tissue was performed under anaerobic conditions. Symbiosomes were isolated by Percoll density gradient centrifugation. Nitrogenase activity (acetylene reduction) was recorded on bacteroids incubated with proline and malate (5 mM).

Transport properties of PBM was determined by incubation of symbiosomes with ^{14}C-proline and ^{14}C-malate. Incubations were stopped using silicone oil filtration technique (Palmieri and Klingenberg, 1979). The protein composition of PBM from symbiosomes of drought stressed and control nodules was investigated by SDS-PAGE.

Results. Bacteroids from drought stressed soybean nodules had a higher nitrogenase activity supported by proline than bacteroids from control plants but drought stress only resulted in a minor increase in the rate of proline uptake by symbiosomes. There was no effect of drought stress on nitrogenase activity supported by malate and the rate of malate uptake by symbiosomes decreased slightly in response to drought stress. The protein composition of PBM was altered as a result of drought stress, which may indicate degradation of PBM proteins.

The results demonstrate that drought stress induces an increased ability of bacteroids to utilize proline to support nitrogenase activity, but PBM may limit an increased supply of proline to the bacteroids, also during drought stress.

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NODULE METABOLISM OF PEA PLANTS WITH VARIED LEAF MORPHOLOGY UNDER DROUGHT.

GONZALEZ E.M.[1], GORDON A.J.[2], MINCHIN F.R.[2], ROYUELA M.[1], APARICIO-TEJO P.M.[1] and ARRESE-IGOR C.[1]
1. Dpto. Ciencias del Medio Natural, Univ. Pública de Navarra. Campus Arrosadía, E-31006 Pamplona, Spain.
2. Dept. Cell Biology, BBSRC Institute of Grassland and Environmental Research. Plas Gogerddan. Aberystwyth, Dyfed SY23 3EB. Great Britain.

Water availability is the major single environmental factor affecting crop yields. A research line to cope with this problem has been the development of pea cultivars with reduced leaf area, which are stated to have a reduced water use. However, the greatly altered morphology poses questions concerning the plant physiology. One important aspect is the relationship between sources and sinks, particularly nodules. As drought is an important limiting factor for nitrogen fixation itself, we have investigated the relationships between the altered foliage morphology and nodule metabolism of pea plants subjected to water stress.

Pisum sativum plants with conventional leaves (cv. Frilene) and semi-leafless (cv. Solara) were inoculated with ***Rhizobium leguminosarum*** bv. *viceae* strain NLV 8 and grown in a mineral medium lacking nitrogen. A gradual drought was imposed when plants were four weeks old. Nodules of control and water stressed plants were sampled at days 0, 7, 14 after the onset of the treatment.

Nodule protein content was significantly reduced in Frilene droughted plants but was maintained for longer in Solara. Little effect was observed in most of nodule enzyme activities when expressed on a protein basis. Nevertheless, sucrose synthase (SS) activity was reduced by 60% in Solara nodules after 14 days drought and, at the same time, a significant sucrose accumulation was found. Fructose did not show any change whilst starch decreased, suggesting remobilisation that led to glucose accumulation in Frilene. Proline was accumulated after two weeks in both varieties. However, total free amino acids increased from the first days under drought. In summary, nodules of both cultivars are affected by drought. The significant reduction in SS activity (a key step in the metabolism of imported sucrose) occurring in Solara may have led to a deficiency in the supply of substrates for the bacteroids. This supports recent work which suggests that the response to drought has a metabolic component and is not due simply to O_2 limitation (Díaz del Castillo et al. 1994) and suggests that our previous observation of a key role of SS in soybean nodule metabolism (González et al. 1995) could be expanded to other species. However, Frilene would be an example of oxygen limitation occurring under drought because of the decline in leghemoglobin content.

Díaz del Castillo L. et al. (1994) Plant Physiol. 106, 949-955
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WHOLE PLANT REGULATION OF N_2 FIXATION IN SOYBEAN: THE ROLE OF C/N RATIO AND NITROGEN TRANSLOCATION.

PARSONS R., MORRIS R.N., BAKER A.
Biological Sciences, University of Dundee, Dundee, DD1 4HN.

In nitrogen fixing plants, nodule growth and activity is consistently matched to plant requirements for nitrogen. Experimental evidence, from studies where plant growth, nitrogen nutrition and nitrogen fixation have been manipulated, are consistent with the operation of an N stat in the symbiotic plant, such that nodule growth and activity are modulated according to the level of N within the plant. The sensing of plant nitrogen may involve the translocation of N, and in particular, we have suggested that the concentration of certain key amino compounds in the phloem sap flowing into nodules may signal plant N status to the nodule (Parsons et al. 1993). In our experiments to manipulate plant growth and nitrogen fixation we have observed increases in nodule activity and growth when nitrogenase is partially inhibited by exposure to $Ar/N_2/O_2$ atmospheres or periodic flushing with O_2. Plants in low light grew slower and had a lower specific nodule fraction as dry weight, suggesting that the reduced demands for N can be met by less nodule material. The most well known influence on nodule growth and activity is combined nitrogen, particularly soil nitrate. Growth of plants in the presence of nitrate reduces specific nodule fraction and specific nitrogenase activity, and at high levels of combined nitrogen, nodulation is totally suppressed. To investigate possible compounds involved in signalling we added $^{15}NO_3$ to the roots of hydroponic plants and collected leaf, root, nodule and xylem sap at various times for GC-MS analysis of amino acids and label incorporation (Table 1).

Time after $^{15}NO_3$ fed (hours)	Leaf asparagine amount : activity umol/g : APE	Nodule asparagine amount : activity umol/g : APE
0	0.14 : 0%	41 : 0%
24	0.51 : 25%	73 : 4.6%
48	1.84 : 48%	104 : 6.6%

Other amino acids and the ureide allantoin were detected in amount and label in extracts from leaf, root, nodule extracts and xylem sap, but asparagine was prevalent and showed the most dramatic changes in concentration. Data of Ohyama and Kumazawa (1979) with soybean and Atkins et al. (1988) with cowpea suggests asparagine is not synthesised in nodules. We interpret our data to support the suggestion that asparagine in nodules is largely derived from phloem delivery, and correlate the increase we observed in plant and nodule asparagine levels, observed when nitrate was fed, with the time course recorded for nitrate inhibition of nodule activity and growth. The exact mechanism by which this signal may influence nodule physiology remains to be determined, but clearly may involve changes in nodule permeability to oxygen and/or alteration of the activity and expression of key enzymes.

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ASPECTS OF C, N, AND O METABOLISM IN NODULES OF SPRINT-2 FIX$^-$ A PLANT-DETERMINED, NON-N_2 FIXING MUTANT OF PEA (*PISUM SATIVUM* L.).

ROMANOV V.I.(1), GORDON A.J.(2), MINCHIN F.R.(2), WITTY J.F.(2), BORISOV A.Y.(3), TIKHONOVICH I.A.(3)
(1)A.N.Bach Institute of Biochemistry, Leninsky prospect 33, Moscow, 117071, Russia. (2)Institute of Grassland and Environmental Research, Plas Gogerddan, Aberystwyth, Dyfed SY23 3EB, UK. (3)All-Russian Institute of Agricultural Microbiology, St. Petersburg-Pushkin, Shosse Podbelsky 3, 189620, Russia.

The plant-determined pea mutant Sprint-2 Fix$^-$ (with mutation in locus symX) and parental line Sprint-2 were compared for selected physiological and biochemical parameters. The Fix$^-$ mutation prevented differentiation of *Rhizobium leguminosarum* bacteria into bacteroid forms and produced large, white, non-fixing nodules. These lacked nitrogenase-linked respiration but had a background rate of CO_2 evolution similar to the normal Fix$^+$ nodules. The cortical structure of the ineffective nodules suggests the existence of an oxygen diffusion barrier and this was supported by a low oxygen concentration in the central region (0.5-3.0%), measured using an O_2 sensitive microelectrode. Sucrose and starch content were similar in normal and ineffective nodules while ononitol content was about 15 times lower in the Fix$^-$ nodules. The distribution of starch was also different in the two nodule types. The activities of glutamine synthetase (GS), sucrose synthase (SS), phosphoenol pyruvate carboxylase and alanine-pyruvate aminotransferase were markedly higher in Fix$^+$ nodules while the activities of pyruvate decarboxylase, alcohol dehydrogenase and glutamate dehydrogenase were higher in Fix$^-$ nodules. The data from immunodetection of host nodule proteins confirmed the reduced levels of SS and almost complete absence of GS and Lb in mutant nodules. There was no significant difference in the amount of nitrogenase component 1 extracted from the microsymbiont of normal and ineffective nodules, but component 2 was hardly detectable in Fix$^-$ mutant.

CHARACTERISTICS OF INEFFECTIVE NODULES INDUCED ON A PEA MUTANT E135 (*sym* 13)

N. SUGANUMA [1], T. HAYASHI [1], K. HAYASHI [1], M. TAMAOKI [1], AND H. KOUCHI [2]
[1] Department of Life Science, Aichi University of Education, Kariya, Aichi 448, Japan; [2] Department of Applied Physiology, National Institute of Agrobiological Resources, Tsukuba, Ibaraki 305, Japan

Isolation of plant mutants that form ineffective nodules provides evidence for the existence of plant genes that are essential for nitrogen fixation. Characterization of such mutants should provide valuable information about the mechanisms whereby host plants control nitrogen fixation activity. We have been characterizing an pea (*Pisum sativum* L.) mutant E135 that forms bacteroid-containing ineffective nodules. The mutation is associated with a monogenic recessive allele, *sym* 13 (Kneen et al., 1990). In E135 nodules, the activities of some of the enzymes involved in carbon and nitrogen metabolism are reduced (Suganuma and LaRue, 1993) and the levels of some polypeptides are altered (Suganuma et al., 1993) but most nodulin genes are expressed (Suganuma et al., submitted). The present study focussed on nitrogenase and leghemoglobin (Lb). E135 nodules often exhibited very depressed levels of acetylene reduction activity, just at the early stages of nodule development, and this activity was then almost undetectable throughout the development of the nodules. However, Western blotting with antiserum against nitrogenase from pea nodules (provided by Dr. T. Bisseling) revealed that the two components of the nitrogenase were synthesized in E135 nodules. By contrast, no increase in the level of heme during nodule development was observed in E135 nodules even though Lb apoproteins were detected throughout the development of nodules by Western blotting with antiserum against soybean Lb. From these results, we hypothesized that a reduced level of functional Lb might be responsible for the ineffectiveness of E135 nodules. We examined the activities of enzymes involved in the synthesis of heme and those of protecting enzymes against active oxygen species, as well as the level of iron in E135 nodules. The activity of δ-aminolevulinic acid (ALA) synthase that produce ALA, a precursor of heme, was detected in the bacteroids from E135 nodules and the activity for formation of ALA from glutamate was detected in the host plant fractions from E135 nodules. Activities of enzymes involved in protecting Lb against active oxygen species, such as ferric-Lb reductase, superoxide dismutase, catalase, ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase, in E135 nodules were not very different from those in effective nodules. Furthermore, levels of iron in E135 nodules were similar to those in effective nodules throughout nodule development. These results suggest that the reduced level of functional Lb in E135 nodules is the result of the absence of nitrogenase activity.

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UPTAKE OF INDOLE-3-ACETIC ACID IN SYMBIOSOMES FROM SOYBEAN (*GLYCINE MAX* L.) ROOT NODULES.

L. ROSENDAHL[1] AND B.U. JOCHIMSEN[2]
[1]Environmental Science and Technology Department, Risø National Laboratory, DK-4000 Roskilde, Denmark; [2]Department of Molecular Biology, University of Aarhus, DK-8000 Aarhus, Denmark.

Introduction. Auxins are a group of phytohormones that may influence legume root nodule formation. Established legume nodules contain higher levels of auxin than the corresponding roots and the most abundant auxin in root nodules is indole-3-acetic acid (IAA) (1). Both symbionts are capable of synthesizing IAA, but it has not been established which of the symbiotic partners is the source of nodule IAA. *Bradyrhizobium japonicum* is able to catabolize IAA (2), thus the bacteroids may constitute an overall sink as well as a source for IAA in soybean (*Glycine max* L.) nodules. The aim of this work was to investigate the capability of IAA transfer between the symbionts across the symbiosome membrane.

Methods. Symbiosomes were isolated from soybean nodules by Percoll density-gradient centrifugation. *In vitro* experiments were performed on uptake of IAA by incubation of symbiosomes with [^{3}H]IAA in a reaction mixture layered on silicone oil. Incubation was terminated by centrifuging the symbiosomes through the silicone oil layer into an underlying perchloric acid layer. Radioactivity in the symbiosome pellet was determined by liquid scintillation analysis.

Results and discussion. Incubation of symbiosomes in [^{3}H]IAA resulted in a time-dependent increase in IAA concentration of the symbiosome pellet, which is an index of uptake. At 15 min, a steady level was reached at 5 pmol IAA in the symbiosomes. This corresponds to an internal IAA concentration of approximately 1 μM in the pelleted symbiosomes (calculated from an estimate of symbiosome volume by incubation of symbiosomes with ^3H_2O). Accumulation of radioactivity in the symbiosomes was competitively inhibited by unlabelled IAA in accord with uptake of IAA in the symbiosomes. Uptake of IAA as a function of IAA concentration suggested the presence of a nonsaturable uptake system. A plot of 1/V versus 1/[S] for IAA indicated a biphasic uptake system and a saturable (carrier mediated) uptake mechanism at [IAA] $\leq$ 200 nM with an apparent K_m of 140 nM and V_{max} of 4 pmol $\cdot$ min^{-1} $\cdot$ mg^{-1} protein. Inclusion of the auxin transport inhibitor 1-N-naphthylphthalamic acid in the reaction mixture resulted in a decrease in IAA accumulation in symbiosomes. This indicates that the carrier-mediated IAA uptake may be ascribed to the presence in the symbiosome membrane of an auxin-efflux carrier system, which directs transport of IAA from the plant cytosol towards the bacteroids.

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ADAPTATIVE RESPONSE OF *Rhizobium tropici* CIAT 899 TO OSMOTIC STRESS.

CASTRO S. AND MARTINEZ-DRETS G.
Biochemistry Division, IIBCE, Ave.Italia 3318, 11600.
Montevideo, Uruguay.

Free-living soil bacteria must adapt to changes in osmotic potential for survival and this problem becomes particularly acute as the soil dries. In response to osmotic stress bacteria produce osmoprotectants (1, 2, 3, 4). It has been reported accumulation of glutamatic acid when cells are osmotically stressed. It has also been proposed that glutamate is produced by the bacteria to act as a counterion for the K+ that accumulates when cells grow under osmotic stress (4, 5). The objective of this research was to study the accumulation and transport of an osmoprotectant : glutamic acid and the expression of enzymes involved in glutamate metabolism in osmotically stressed *Rhizobium tropici* CIAT 899. Sodium chloride, sucrose and polyethylene glycol (PEG) were used as osmolites. Intracellular glutamate was determined by HPLC analysis using o-phthaldialdehyde as a pre-column fluorescense derivatization reagent. Two to six fold accumulation of glutamate was found when cells were osmotically stressed. Neomycin (an inhibitor of protein synthesis) did not alter glutamate production, suggesting that *de novo* synthesis were not involved in the response to osmotic stress. Glutamate transport was also induced in hiperosmotic conditions. Glutamine synthetase (GS); glutamate synthase (GOGAT); glutamate dehydrogenase (GDH); aspartate (AspAT), alanine (AlaAT), aromatic (ArAT), and branched-chain amino acid (BcAT) transferases acitivity were determined. GS and GOGAT activities were induced in bacteria exposed to osmotic shock. The induction of GOGAT was lost when neomycin was used. GDH inhibition was detected when bacteria were subjeted to hiperosmolarity for long periods of incubation. In addition, GDH activity was reduced when K+ was added to incubation mixture. BcAT, ArAT and AspAT activities were induced in bacteria exposed to osmotic shock; however, no ArAT activity was induced when PEG was used as osmolite. No induction of AspAT acitivity was detected in the presence of neomycin. Our results show that glutamate was accumulated when *Rhizobium* cells were osmotically stressed. Glutamate uptake was also induced when cells were stressed with NaCl. Enzymes of GS-GOGAT pathways, responsable of glutamate biosynthesis, were induced when cells were exposed to osmotic shock with all the tested osmolites. However, GDH activity was reduced when cells were stressed with NaCl.

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THE INVESTIGATION OF METABOLIC CHANGES IN *RHIZOBIUM MELILOTI* STRAINS BY MEANS OF ^{31}P ,^{15}N - NMR SPECRTROSCOPY.

NIKOLAEV B.P., PROVOROV N.A., SIMAROV B.V., SCHLAKOV A.M.
Research Institute of Highly pure biopreparates, Pudogskaya 7, St-Petersburg, 197110, Russia; All-Russian Research Institute for Agricultural Microbiology, Podbelsky Shaussee 3, St-Petersburg Pushkin 6, 189620, Russia.

Biochemical changes of low molecular phosphorous and nitrogen methabolites of symbiotic bacteria Rhizobium meliloti with relative nitrogen fixing ability were studied by ^{31}P, ^{15}N -NMR fourier spectroscopy. Initial steps of methabolism were investigated by measurement oflevels and protonation states of glucose-6-phosphate, inorganic phosphate, $^{15}NH_4^+$ and glutamate in cell suspension nodules in glycolys and active respiration conditions.There were some correlation between symbiotic and fixing efficiency and characteristics of dynamics of methabolic trasformation of ^{31}P , ^{15}N -substrates. Nitrogen-fixing and non-symbiotic strains have been appeared to differ in isotope ^{15}N distribution in cell envelopes by NMR data. Conversion of glucose-6-phosphate into pyruvate by aerobic non-fixing strains CXM-1 follows the slow kinetics compared with highly effective strain T798 .

DIFFERENT PATHWAYS OF AMMONIA ASSIMILATION IN LEAVES AND ROOTS OF KIDNEY BEAN

SADUNISHVILI T.A., NUTSUBIDZE N.N.
Institute of Plant Biochemistry, Georgian Academy
of Sciences, Tbilisi, 380059, Republic of Georgia

Coenzyme specificity, multiple molecular forms and intacellular distribution of enzymes involved in ammonia assimilation has been studied. It was shown, that glutamate dehydrogenase, glutamine synthetase and glutamate synthase available in leaves and root of young kidney bean seedlings show different specificities towards coenzymes as well as different spectrum of multiple molecular forms (Sadunishvili, Nutsubidze 1985; 1986). The changes of glutamine synthetase, glutamate synthase and glutamate dehydrogenase specific activities, protein and ammonia accumulation during ^{15}N-ammonia assimilation as well as the amounts of free amino acids and the kinetics of the incorporation of ^{15}N into them were investigated. On the basis of the data obtained under usual conditions and in case of glutamine synthetase inhibition by L-metthionine sulfoximine the pathways of ammonia primary assimilation in leaves and roots has been established. In leaves it occurs via the combined action of glutamine synthetase and glutamate synthase (Sadunishvili et al. 1989). In roots, in addition to glutamate synthase cycle, glutamate dehydrogenase is also involved in ammonia primary assimilation.

As for ammonia excess, the pathway of its detoxication in both leaves and roots is via glutamate dehydrogenase (Sadunishvili et al. 1993).

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EFFECTS OF LIGHT DURATION AND NITROGEN SOURCE ON THE ACTIVITY AND COMPOSITION OF GLUTAMINE SYNTHETASE MOLECULAR FORMS IN *AZOLLA-ANABAENA* SYMBIOSES.

SOF'IN A.V., KOMINA O.V., SHATILOV V.R.
A.N.Bach Institute of Biochemistry, Leninsky
prospect 33, Moscow, 117071, Russia.

The *Azolla mexicana-Anabaena azollae* symbiosis has been maintained in nitrogen fixing conditions with a photoperiod of 12 hours. At the end of a dark period in host-plant leaves only cytoplasmic glutamine synthetase (GS, EC 6.3.1.2) was found. After 1 hour of illumination the chloroplastic GS appeared. Its activity increased rapidly during first 2h of illumination and remained then at a constant level to the end of light period. We suggested that such a rapid appearance of an active chloroplastic GS can be due to both an activation by light preexisting enzyme molecules and the synthesis of the new ones. After 5h of the light period unknown form of GS was found in the leaves of *A.mexicana*. The activity of this GS form increased and by the end of light period exceeded of that of chloroplastic GS.

In the presence of nitrate (5 mM) in the medium during 7 days of cultivation of *A.filiculoides-A.anabaena* association the specific host-plant GS activity increased up to 50%. In the presence of ammonium (1 mM) only a transferase GS activity increased in 2 times while a biosynthetic one did not change practically. Also, ammonium induced the appearance *de novo* of the cytoplasmic GS in the *A.filiculoides* leaves.

CHARACTERISATION OF THE NODULE-SPECIFIC REPRESSION OF L-ASPARAGINASE GENE TRANSCRIPTION IN LEGUMINOUS NODULES

VINCZE E., REEVES J.M., REYNOLDS P.H.S.
Plant Improvement Division, The Horticulture and Food Research Institute of New Zealand
Ltd, Private Bag 11030, Palmerston North, New Zealand

Upon the establishment of an effective nitrogen-fixing symbiosis in amide-transporting plants, the enzymatic activity and transcript levels of L-asparaginase are dramatically decreased. This decrease in L-asparaginase activity is essential for the correct functioning of the *Rhizobium* -legume symbiosis in which asparagine, synthesized from recently fixed nitrogen, is exported to aerial parts of the plant for use in growth and development. Concomitant with this decrease in L-asparaginase transcript, a DNA-binding protein has been detected in the nodules. The DNA-binding activity was shown to interact with a 59 bp sequence proximal to the transcription start site. Within this sequence, a CTAAAAT direct repeat and a ACTGT/TGTCA incomplete inverted repeat were implicated in the binding of the protein to the DNA (Vincze et al. 1994).

DNA-protein complex formation between the 59 bp TATA proximal sequence and protein extract from *Lupinus angustifolius* or *Lotus corniculatus* nodules of different developmental stages showed the same pattern. This suggests that the appearance of a DNA-binding protein associated with repression of the L-asparaginase gene is developmentally controlled in both lupin and lotus nodules. Experiments using transgenic lotus plants have indicated that the repressor protein binding site from lupin is functional in lotus nodules and protected against overexpression of an introduced L-asparaginase gene. Nodulation tests were performed using different *Fix*$^-$ *Tn5* mutants of *R. loti* (Chua et al., 1985). Protein was isolated from the nodules of these plants, and gel retardation assays were performed. The L-asparaginase gene repressor protein was not detectable in ineffective nodules induced by one of the *Noi* symbiotic mutants of *Rhizobium loti* (PN236). Complementation of the PN236 mutation with the pLAFR1 cosmid carrying the appropriate wild type *Rhizobium* sequence restored the DNA-protein complex formation ability. The *Rhizobium loti* mutant analysis is consistent with the repressor protein being of bacteroid origin or being induced by some bacterial factor. A *Tn5* rescue experiment has been performed, the sequences bordering the transposon determined and the exact location of the insertion mapped from the PN236 mutant. Currently we are sequencing the complementing wild-type *Rhizobium* DNA fragment from the complemeting cosmid clone to identify the functional gene.

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ASPARTATE AND ALANINE TRANSPORT ACROSS THE SYMBIOTIC MEMBRANES FROM SOYBEAN NODULES

LYNNE WHITEHEAD, SUSAN YOUNG, LESLEY SCHULLER,
DAVID A. DAY
Division of Biochemistry and Molecular Biology, Faculty of Science,
Australian National University, Canberra, ACT 0200, Australia.

The characteristics of aspartate and alanine transport across the peribacteroid and bacteroid membranes from soybean nodules were studied. Bacteroids were found to have an aspartate uptake system with dual kinetics, involving both passive and active components. The active component (K_m = 27.5 µM, V_{max} = 5.14 nmol mg protein^{-1} min^{-1}) was not stimulated by ATP, but it was strongly inhibited by cyanide and appeared to be dependent on a proton gradient across the bacteroid membrane. There was no evidence for an aspartate transporter on the peribacteroid membrane. Aspartate uptake by bacteroids was competatively inhibited by glutamate (K_i= 9.7 µM). However, aspartate did not have the same inhibitory effect on bacteroid glutamate uptake. Aspartate and glutamate were found to be exchanged across the bacteroid membrane. No convincing evidence was found for a similar exchange across the peribacteroid membrane nor for the exchange of aspartate and malate. Alanine transport in bacteroids showed carrier mediated kinetics similar to that of aspartate (K_m = 14.7 µM, V_{max} = 3.05 nmol mg protein^{-1} min^{-1}). Transport across the peribacteroid membrane was by diffusion only. These results suggests that there are high affinity transporters for amino acids on the bacteroid membrane whereas the peribacteroid membrane is barely permeable to these substrates. This make it highly unlikely that either amino acid is a major energy source for bacteroids within active soybean nodules. The kinetics of these transporters suggests a scavenging role rather than a mass transport role. In addition, the exchange data indicates that aspartate and malate are not exchanged across the peribacteroid membrane.

IS THE SYMBIOTIC NITROGEN FIXATION OSMOREGULATED BY REVERSIBLE CONTRACTION OF CELLS IN THE LEGUME-NODULE INNER-CORTEX?

DREVON J.J. (1), DERANSART C. (1), FLEURAT-LESSARD P.(2), JAILLARD B. (1), NDJIONDJOP M.N. (1), PAYRE H. (1), RIBET J. (1), ROY G. (1), SERRAJ R. (3).(1) Inst. National de la Recherche Agronomique, Place Viala, 34 000 Montpellier, FRANCE. (2) Centre National de la Recherche Scientifique, UA 574, Station Biologique de Beau-Site, 86 000 Poitiers, FRANCE. (3) Universite Cadi Ayyad, BP S15, Marrakech, MAROC.

The reduction of molecular nitrogen in legume root-nodule requires a low intronodular O_2 concentration which may result from the combination of a high nitrogenase-linked respiration in the infected zone and a low conductance to O_2 diffusion in the nodule cortex. In order to better understand this control of O_2 diffusion we developed a method to measure nodule conductance to O_2 diffusion (g_{no}), and performed cytologic observations of the cortex of nodules differing in their g_{no}.

By measuring the response of the O_2 uptake of soybean nodulated-roots to the external pressure of O_2 (pO_2), we show that the g_{no} decreases rapidly in response to an increase in pO_2, and increases in response to a decrease in pO_2, although more slowly. Moreover, the phosphorus deficiency induces the formation of nodules having a higher g_{no} than the one of nodules of soybean plants grown with sufficient phosphorus.

The computerized morphometric analysis of the cortical cells of transversal nodule cuts shows than the decrease in g_{no} induced by an excess of O_2 or NaCl is associated with a decrease in the area and an increase in the elongation of cells in the inner-cortex, i.e. in cell layers between the infected zone and the vascular traces.

Protoplasts of these inner-cortex cells could be obtained by digestion of nodule cuts by cellulolytic and pectinolytic enzymes, and characterized by large strach deposits. Moreover cyanide insensitive respiration was found in mitochondria isolated from nodule-cortex.

Our results lead us to hypothesize that the energetic metabolism linked to the nitrogenase activity of legume root-nodule is regulated by variations of the nodule conductance to oxygen diffusion due to contraction-expansion of postulated osmocontractil cells in the nodule inner-cortex. Further work consists in cellular and molecular characterization of inner-cortex cells of legume root-nodule

HOW TEMPERATURE AFFECTS THE METABOLISM OF SOYBEAN NODULES

KUZMA M. M., LAYZELL D. B.
Biology Department, Queen's University, Kingston, Ont., Canada, K7L 3N6

In legume nodules the concentration of O_2 in the infected cells (Oi) regulates respiration rate and nitrogenase activity. This study investigated the effects of temperature on the relationship between Oi and respiration rate in N_2-fixing nodules of soybean (*Glycine max* L. Merr). Intact nodules attached to the root were studied at 15, 20 and 25°C using nodule oximetry. An increase or a decrease in nodule temperature resulted in an increase or a decrease, respectively, in nodule central zone respiration rate that could be supported at a given Oi. Since Oi limits the respiration rate of legume nodules, but is determined by the balance between the respiration rate and nodule permeability to O_2 diffusion, an increase in temperature caused a rapid decrease in Oi and a decrease in temperature caused a rapid increase in Oi. These results were integrated with the previous gas exchange study of intact nodulated roots of soybean (Kuzma and Layzell, 1994) to explain how temperature affects the metabolism of soybean nodules. The effect of temperature-induced changes in Oi on nodule activity was found to be dependent upon the physiological state of the nodule during the temperature treatment. For example, nodules inhibited by the oximetry treatment maintained a similar respiration rate and nodule permeability at all temperatures, but varied widely in their Oi and the degree to which nodule metabolism was limited by O_2. Alternatively, uninhibited control nodules used in the gas exchange study responded to temperature-induced changes in Oi by altering their permeability to O_2 diffusion, thereby returning Oi to levels which supported respiration rates that were not limited by O_2. This interaction between temperature effects on nodule metabolism and the physiological state of the nodule may account for some of the apparent inconsistencies among reports in the literature that describe temperature effects on nodule metabolism and N_2 fixation in legumes.

Kuzma MM, Layzell DB (1994) Acclimation of soybean nodules to changes in temperature. Plant Physiol **106**: 263-270

MECHANISMS FOR SHORT-TERM (15 MIN) CHANGES IN THE OXYGEN DIFFUSION BARRIER OF LEGUME NODULES.

MINCHIN F.R.(1), IANNETTA P.P.M.(2), JAMES E.K.(3),
SPRENT J.I.(2), THOMAS B.J.(1), WITTY J.F.(1).
(1)IGER, Plas Gogerddan, Aberystwyth, Dyfed SY23 3EB, UK. (2)Department of Biological Sciences, University of Dundee, Dundee DD1 4HN, UK. (3)Plant Sciences Laboratory, University of St Andrews, St Andrews KY16 9TH, UK.

The combining of physiological measurements with mathematical analyses has provided extensive evidence for a variable oxygen diffusion barrier in legume nodules. However, there is still controversy as to its location and structure. Most structural analyses have involved changes over time periods of 2 h to 28 d, whilst barrier changes can occur within 10-15 min. This poster provides evidence for short-term structural changes within the cortex of nodules. Sections through unstressed lupin nodules show several unoccluded intercellular spaces between the thick-walled cells of the mid-cortex. A 15 min exposure to 50% O_2 produces a marked increase in space occlusions as shown by silver-enhanced immunogold labelling (with MAC 236) of an intercellular glycoprotein. It has recently been suggested that the inner cortical cells of soybean nodules could act in an osmocontractile manner; collapsing during stress to release water into the intercellular spaces. Support for this hypothesis is provided by X-ray microanalysis of freeze-shattered soybean nodules. This shows a marked increase in Ca^{++} and a loss of vacuolar K^+ from the inner cortical cells occurring within 15 min of nodule disturbance (root shaking). Some ability to vary O_2 diffusion resistance may also exist within the infected cells; as suggested by two recent mathematical models. An intriguing addition to this idea is the observation of intercellular glycoprotein within the infected region of unstressed soybean nodules. In conclusion, the O_2 diffusion "barrier" may not be a single structural feature. Instead, it may be a composite of metabolic and structural changes occurring in the infected region, the inner cortex and the mid-cortex. If this is confirmed it will then be necessary to understand the interrelationships between O_2 diffusion control at these different levels.

THE LEGUME SYMBIOSIS: C-LIMITED BACTERIA LIVING WITHIN O_2 LIMITED PLANT CELLS?

ORESNIK I.J.[1], ATKINS C.A.[2], LAYZELL D.B.[1]
[1] Biology Department, Queen's University, Kingston, Ontario, Canada K7L 3N6
[2] Botany Department, University of Western Australia, Nedlands 6009,Western Australia

Under normal physiological conditions nitrogenase activity in soybean (*Glycine max* L. Merr.) is limited by O_2 concentration and it is widely believed that the O_2 limitation occurs in the N_2 fixing bacteria, not in the plant cells. To test this hypothesis, a non aqueous method was developed to separate and quantify the pools of key metabolites (ATP, ADP, AMP, and malate) in the plant and bacteroid compartments of nodules rapidly frozen at various degrees of O_2 limitation. The adenylate energy charge ((ATP+0.5*ADP)/(ATP+ADP+AMP)) in the bacteroid compartment was high (0.81±0.04), indicative of aerobic cells, and unaffected by the degree of O_2 limitation in the nodule. However in the plant compartment, the AEC was 0.80±0.04 under supra optimal O_2 and declined sharply to 0.50±0.03 under severe O_2 limitation. Under optimal conditions for nitrogenase activity, the AEC of the plant compartment was 0.59±0.03. These data indicate that in soybean nodules, the O_2 limitation was in the plant compartment, not in the bacteroid compartment as previously thought.

To test how O_2 limitation may regulate nitrogenase activity *in vivo*, the concentration of malate was determined in the plant and bacteroid compartments. Compared to the control nodules, it was found that nodules with lower nitrogenase activity caused by greater O_2 limitation, had higher malate concentrations in the plant compartment and lower bacteroid compartment malate concentrations. There was a negative correlation (r^2= 0.70) between the plant compartment malate concentration and *in vivo* nitrogenase activity whereas there was a positive correlation (r^2=0.78) between the bacteroid malate concentrations and *in vivo* nitrogenase activity from nodules which were inhibited by varying the oxygen concentration around the nodulated rhizosphere. We propose that the O_2 limitation in the plant compartment, reduces ATP availability thereby reducing the supply of carbon substrates which are available to the bacteroids.

EFFECTS OF THE MODIFICATION OF THE OXYGEN CONCENTRATION IN *Rhizobium etli* AS A RESULT OF THE EXPRESSION OF THE *Vitreoscilla* GLOBIN GENE (*vgb*).

RAMIREZ M.[1], VALDERRAMA B.[1], ENCARNACION S.[1], SUAREZ R.[1], SOBERON M.[2], MORA J.[1], and HERNANDEZ G.[1]
[1]Centro de Investigación sobre Fijación de Nitrógeno. UNAM.
Departamento de Ecología Molecular. Ap. Postal 565-A.
Cuernavaca, Mor. México.
[2]Instituto de Biotecnología. UNAM. Ap. Postal 510-3, Cuernavaca, Mor.

The oxygen concentration is a major regulatory factor for endosymbiotic nitrogen fixation gene expression. The endosymbiont rhizobia adapt their obligatory respiratory metabolism to a microaerobic environment.

The gram negative bacteria: *Vitreoscilla* sp, produces a bacterial hemoglobin (Vhb) in large quantities in response to hypoxic environments. Vhb is coded by the *vgb* gene, which has been cloned and characterized and it has been expressed, driven by its own promoter, in *E. coli* and other gram negative bacteria in an oxygen dependent manner (Dikshit et al., 1990; Khosla et al., 1990).

To achieve the modification of the intracellular oxygen concentration in *Rhizobium etli*, the *Vitreoscilla vgb* gene, was cloned into the stable RK2 derivative pTR101 (Weinstein et al., 1992) (plasmid pMR04), and expressed in *R.etli* CFN42 strain. We have initiated to investigate the effect of an altered oxygen concentration, which might result from the presence of Vhb, in the expression of oxygen regulated nitrogen fixation genes. The *explanta* expression (determined as b-galactosidase activity in strains with *lacZ* gene fusions) of two of the *R. etli* reiterated *nifH* genes: *nifHa* and *nifHc* (Quinto et al., 1982) which are known to be induced in low oxygen concentrations (Valderrama et al., this book) is increased in strains bearing the pMR04(+*vgb*) when incubated in very low oxygen concentration (0.25%-0.75%). The Vhb oxygen binding might provide the adequate internal oxygen concentration for the *nifH* gene expression when oxygen in the atmosphere is very low. The *R. etli* CFN42/pMR04(+*vgb*) strain shows increased nodulation and nitrogen fixation (as compared to the wild type strain without Vhb) at early stages of the symbiosis with beans (*Phaseolus vulgaris*) which might lead to a higher plant growth and earlier pod production.

Our results indicate that the expression of an heterologous hemoglobin gene in *Rhizobium* provides a system to modulate the intracellular oxygen concentration, which is a key determinant for the regulation of the expression of essential symbiotic genes.

Dikshit et al. (1990) Nucl. Acids Res. 18, 4149-4155.
Khosla et al. (1990) BioTechnology, 8, 849-853.
Quinto C et al. (1982) Nature 299, 724-726.
Valderrama et al., this book
Weinstein et al. (1992) J. Bacteriol. 174, 7486-7489.

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EFFECTS OF TEMPERATURE ON LEGHEMOGLOBIN FUNCTIONING AND OXYGEN CONDITIONS IN NODULES

TOPUNOV A.F. (1), KUZMA M.M. (2), THUMFORT P.P. (3), ATKINS C.A. (3), LAYZELL D.B. (2)
(1) A.N.Bach Institute of Biochemistry, Russian Academy of Sciences, Moscow, Russia; (2) Queen's University, Kingston, Canada; (3) University of Western Australia, Nedlands, Australia

It is known that temperature influence many parameters of legume nodules. Effects of short-term temperature changes on N_2-fixation were studied recently (Kuzma, Layzell, 1994). It was proposed that changes of nodule temperature caused changes in the rate of respiration. Temperature induced changes in respiratory level can be connected with oxygen conditions in infected nodule cells and therefore with leghemoglobin (Lb) functioning. For examination of this relation effects of temperature on Lb were studied. The affinity of soybean Lb for oxygen was measured at 15, 20 and 25°C. The K_c values at these temperatures were 44, 60 and 78 nM O_2 respectively. A difference in 10°C resulted in 1.77-fold difference in K_c. These values were used to calculate the infected cell O_2 concentration. They were also compared with data of fractional oxygenation of leghemoglobin measured using oxymetry method. Both these data were in good agreement with one another. Data of Lb affinity for oxygen were also used for mathematical approaches of effects of temperature both on oxygen conditions in infected nodule cells (including resistance of an infected cell to O_2 diffusion) where mathematical approach of Thumfort et al. (1995) was used, and on the cortical diffusion barrier resistance. Resistance of an infected cell to O2 diffusion predicted from the infected cell model indicates the importance of a cortical barrier in the control of O_2 diffusion. At the same time there may still be a need to regulate O2 diffusion at the infected cell level.

Kuzma MM, Layzell DB (1994) Plant Physiol. 106, 263-270.
Thumfort PP et al. (1994) Plant Physiol. 105, 1321-1333.

ROOT NODULE FERRIC LEGHEMOGLOBIN REDUCTASE WHICH CONTAINS A NON-HEME IRON

ZHIZNEVSKAYA, G.Y.. BORODENKO, L.I.,
KUDRYAVTSEVA N.N.
K.A Timiriazev Institute of Plant Physiology Russian Academy of Sciences, 35 Botanischeskaya, Moscow 127276, Russia

Iron-containing proteins are abundant in the root nodules of leguminous plants. The non-heme iron is found in nitrogenase components of bacteroids. Non-heme iron storage protein ferritin takes part in redistribution of iron within the nodule to heme (of leghemoglobin, catalase and peroxidase) and possibly nitrogenase.

We used the technique described for the ferredoxin isolation to separate leghemoglobin, peroxidases and catalase from the root nodule cytoplasmatic fractions, containing non-heme iron determined after splitting with 2N HCl. The brown coloured non-heme iron protein fraction was isolated from the root nodules of *Lupinus luteus* L., *Soya hispida* L. and *Vicia faba* L., to be more abundant in soybean nodules possessing the highest amount of leghemoglobin.

The non-heme iron protein has been purified from the root nodules of yellow lupin as a single brown band with r.e.m. of 0.5 on PAGE, with molecular wt 100,000, two atoms of non-heme iron for one mole of protein and Fe:Mn ratio near 4:1 when analysed by atomic absorption technique, and it contains FAD, as was shown fluorometrically. Manganese and iron signals were found using electron-paramagnetic resonance technique in this non-heme iron protein.

The non-heme iron protein is rapidly oxidized by ferricyanide and appeared to be a NAD (P)H dependent reductase capable of reducing cytochrome C, leghemoglobin, ferricyanide and dichlorophenolindophenol.

The nature of iron-binding environment inside protein needs to be determined.

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Zhiznevskaya GYA et al (1969) In Abstracts of the Eleventh International Botanical Congress, p. 247, Seattle, WA, USA.

ENERGETICS AND PHYSIOLOGY OF NITROGEN FIXATION IN FREE-LIVING *AZORHIZOBIUM CAULINODANS.*

BOOGERD F.C., PRONK A.F., STOUTHAMER A.H.,
AND VAN VERSEVELD H.W.
Department of Microbiology, Institute for Molecular Biological Sciences,
BioCentrum Amsterdam, Vrije Universiteit, de Boelelaan 1087, 1081 HV
Amsterdam, The Netherlands

Free-living growth of *Azorhizobium caulinodans* was extensively studied in carbon-limited chemostat cultures (I,II,IV) and pH-auxostat cultures (I,III) of the wild type and various mutant strains.

I. *A.caulinodans* was proven to be a true diazotroph. The relatively high nicotinate concentration (300 mM) reported to be required for growth on N_2 could be reduced to submicromolar (< 1 mM) levels in chemostat cultures before washing out occurred. In pH-auxostat cultures it was established that at such suboptimal nicotinate levels the specific growth rate (m) was directly proportional to the nicotinate concentration.

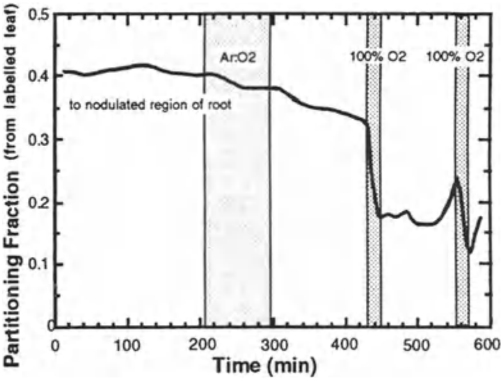
II. Determination of the *in situ* reduction levels of the cytochrome *c* pool directly in steady-state chemostat cultures and the *in vitro* cytochrome *c* oxidase activity of resting cell suspensions revealed that these two parameters were negatively correlated: a low cytochrome *c* oxidase activity was linked to a high reduction level of the cytochrome *c* pool during growth. Comparison of the results obtained with the wild type and the *aa*$_3$ single or *aa*$_3$/*d* double mutant strains grown on NH_4^+ learned that (i) an alternative cytochrome *c* oxidase is active in the *aa*$_3$ mutant strain cultures growing at a dissolved O_2 tension (DOT) of 0.5%, which is hardly present under fully aerobic conditions, and (ii) cytochrome *aa*$_3$ is used in wild-type cultures growing under full aerobiosis.

III. The specific growth rate for growth on N_2 as measured in pH-auxostat cultures was found to be dependent on the DOT and the complement of terminal oxidases present; m^{max} of the wild type and the *aa*$_3$ single or *aa*$_3$/*d* double mutant strains decreased from 0.16 to 0.12 h^{-1} and from 0.19 to 0.10 h^{-1}, respectively, when the DOT was increased from 0.5 to 4.0%.

IV. Determination of the nitrogenase-mediated H_2 production, by means of a combined chemostat-mass spectrometer set-up, in chemostat cultures of a hydrogenase-negative mutant growing at decreased partial pressures of N_2 revealed that the decline in growth efficiency could be fully explained by the observed increase in the H_2/N_2 ratio.

ARE TRANSPORT SOLUTE FLUXES INVOLVED IN AN OSMOTIC MECHANISM CONTROLLING SOYBEAN NODULE PERMEABILITY?

BROWN, S.M., WALSH, K.B., THORPE, M.[1] and MINCHIN, P.E.H.[1]
Dept. of Biology, Central Queensland Uni., Rockhampton, 4702, Australia.
[1]HortResearch, Box 31 313, Lower Hutt, New Zealand.



In soybean, change in the radial length of a cortical osmocontractile cell layer may afford O_2 protection to nitrogenase. A decrease in water mobility within the nodule cortex was observed using 1H NMR spectroscopy (collaboration with Uni of Dundee) during 3 h treatments of girdling and $Ar:O_2$. MAC 265 - glycoprotein increased ca. five fold following these stress treatments, as measured by ELISA. Nodule diameter, as assessed by LVDTs, was responsive to plant water potential, and increased following girdling but not $Ar:O_2$. Thus changes in nodule permeability are not reflected in overall nodule dimension. These results are consistent with a decrease in cortical permeability following stress treatment due to the filling of cortical air spaces with water of decreased mobility (i.e. bound to glycoprotein) rather than a closure of intercellular spaces by cell expansion. Apoplastic tracers should therefore be able to penetrate to the infected zone of nodules of low permeability.

The fraction of recent photosynthate which was partitioned to the nodulated root (C-11 method, 1) was not affected by preventing N_2 fixation by $Ar:O_2$ treatment, but import to the root was halted by girdling and by nitrogenase inactivation with 100% O_2. Non-nodulated roots were not affected by 100% O_2 treatment. We conclude that the sucrose unloading process is not under a turgor control, influenced by the concentration of N solutes (2), but is responsive to a metabolic cue. Nodule soluble sugar (anthrone) content increased following $Ar:O_2$ treatment and declined following girdling, while ureides depleted under both treatments, with a more rapid decrease from the apoplast than symplast under $Ar:O_2$. The combined sugar and ureide solute pool increased to 140%, and decreased to 75%, of initial levels following 3 h of $Ar:O_2$ and girdling treatment respectively. This evidence is not consistent with a control of nodule permeability based on an osmotic mechanism involving a balance between the export and import solutes.

1. Thorpe MR and Minchin PEH (1991) J. Exp. Bot. 42, 461-468
2. Walsh K.B. (1995) Soil Biol. Biochem. 27, 637-655

THE RESPONSE OF N_2 FIXING *SESBANIA ROSTRATA* AND *GLYCINE MAX* TO ELEVATED PARTIAL PRESSURES OF CO_2

GODBER I.M., PARSONS R.
Department of Biological Sciences, University of Dundee, Dundee, DD1 4HN.

Sesbania rostrata and *Glycine max* plants were grown under conditions of ambient and elevated partial pressures of CO_2 (36, 56 and 73 Pa) in closed chambers within a glasshouse for 120 days. Responses of the plants were measured in terms of leaf N content, photosynthetic response, nodule fraction and specific nodule activity.

Leaf N levels measured on a dry weight basis declined from 2.8 to 1.9 % N at 36 and 73 Pa respectively in sesbania and from 1.9 to 1.6 % N in soybean. These results confirm data already obtained for a variety of C3 and C4 plants (Conroy and Hocking, 1993). It is suggested that this is due to a reduction of N in leaf proteins and/or a proportional increase in starch and other carbon compounds.

Gas exchange analysis on sesbania using a CIRAS IRGA allowed us to plot A/Ci curves where the response of assimilation rate (umol CO_2m^{-2} s^{-1}) is determined a range of leaf internal CO_2 concentrations (Pa). The initial slope of the line indicates the carboxylation efficiency of Rubisco. A slight decline in the initial slope was observed in plants grown in conditions of high CO_2, which may be due to the allocation of N away from Rubisco.

Acetylene reduction assays on enclosed sesbania roots (intact plants) showed specific nodule activities of 96, 150 and 146 umol C_2H_4 g_{-1} DW hr_{-1} at growth CO_2 of 36, 56 and 73 Pa respectively. However the nodule fraction was found to decrease from 6.2% at 36 Pa to 4.9% at 56 Pa and 4.4% at 73 Pa in plants with no stem nodules. This indicates that the regulation of nodule growth and activity may be influenced differently by elevated partial pressures of CO_2. We conclude that rising atmospheric CO_2 levels will influence the growth and relative competitive advantage of nitrogen fixing plants to non-nitrogen fixing plants in many ecosystems.

Conroy J. and Hocking P. (1993) Physiologia Plantarum, 89, 570-576.

PHOTOSYSTEM II AND OXYGEN REGULATION IN *SESBANIA ROSTRATA* STEM NODULES.

JAMES E.K.[1], IANNETTA, P.P.M.[2], NIXON P.[3], WHISTON A.J.[1], PEAT L.J.[1], CRAWFORD R.M.M.[1], SPRENT J.I.[4], BREWIN N.J.[5]
[1]Plant Sciences Laboratory, Sir Harold Mitchell Building, University of St. Andrews, St. Andrews, Fife, UK. [2]Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK. [3]Wolfson Laboratories, Department of Biochemistry, Imperial College, London SW7 2AY. UK. [4]Department of Biological Sciences, University of Dundee, Dundee DD1 4HN, UK. [5]John Innes Institute, Colney Lane, Norwich NR4 7UH, UK.

Stem nodules on the tropical wetland legume *Sesbania rostrata* Brem. contain chloroplasts in the uninfected, cortical cells surrounding the N_2-fixing tissue. Leaves, stem and root nodules were examined for the presence of the thylakoid membrane proteins D1 and D2 (polypeptides of the reaction centre core complex) and PsbO (33 kDa extrinsic protein), which are involved in the oxidation of water and the release of O_2. Immunoblotting showed these to be present in extracts of leaves and stem nodules but absent from root nodules. Immunogold labelling confirmed their presence on thylakoids of leaf and stem nodule chloroplasts, with labelling of stem nodules being most intense in well-developed chloroplasts in the mid-cortex. Leghaemoglobin was localised in infected cells adjacent to the cortex and nitrogenase was also detected in bacteroids. This suggests that, despite the possibility of O_2-evolution, the infected cells of stem nodules still maintain a very low pO_2. Nodule extracts examined by ELISA and immunoblotting using the monoclonal antibody MAC265 showed greatly enhanced expression of a 240 kDa glycoprotein in stem compared to root nodules. Material containing the MAC265 antigen was present in intercellular spaces throughout the cortex of stem nodules, particularly in the chloroplast-rich mid-cortex, but was considerably less abundant in root nodules. It is suggested that stem nodules have increased levels of intercellular glycoprotein either to prevent damaging levels of photosynthetically-derived oxygen from cortical chloroplasts entering the infected tissue and/or to compensate for the aerial nature of the stem nodules which exposes them to full, atmospheric pO_2 levels.

LOCALISATION OF PHOTOSYSTEM II COMPONENTS IN SYMBIOTIC AND FREE-LIVING HETEROCYSTOUS CYANOBACTERIA.

JAMES E.K.[1], ROWELL P.[2], NIXON P.[3], CRAWFORD R.M.M.[1], SILVESTER W.B.[4]

[1]Plant Sciences Laboratory, Sir Harold Mitchell Building, University of St. Andrews, St. Andrews, Fife KY16 9TH, UK. [2]Department of Biological Sciences, University of Dundee, Dundee DD1 4HN, UK. [3]Wolfson Laboratories, Department of Biochemistry, Imperial College, London SW7 2AY. UK. [4]Department of Biological Sciences, University of Waikato, New Zealand.

Heterocystous cyanobacteria prevent photosystem II (PSII) reactions from damaging O_2-labile nitrogenase by compartmentalising the former into vegetative cells, and the latter into heterocysts with thick walls (Gallon, 1992). However, in this study we show that PSII components may still be present in heterocysts, although they are probably non-functional. Immunogold labelling and immunoblotting has been used to localise the thylakoid membrane proteins D1 and D2 (polypeptides of the reaction centre core complex which receive and transfer electrons to plastoquinone) and PsbO (the 33 kDa extrinsic protein of the oxygen-evolving complex). These are major proteins in PSII involved in the oxidation of water and the release of O_2. PsbO was shown to be present in vegetative cells and heterocyst preparations (the latter also expressing the nitrogenase Fe-protein) from *Anabaena cylindrica* and, PsbO, D1 and D2 were localised on thylakoids of *A.cylindrica*, *Nostoc* and symbiotic *Nostoc* in *Gunnera manicata.* D2 was particularly abundant in vegetative cells from all species, but was also present in heterocysts which were also labelled abundantly with antibodies against nitrogenase Fe amd Mo-Fe proteins. D1 was the least apparent of the PSII proteins and found only in vegetative cells, whereas PsbO was localised in both heterocysts and vegetative cells. It is concluded that non-symbiotic vegetative cells are likely to contain the full apparatus of PSII proteins involved with oxygen evolution and this may also be true of heterocysts and symbiotic cyanobacteria (although to a lesser extent). However, in heterocysts and *Gunnera* there is no evidence that the O_2-evolving proteins are functional, as PSII does not operate, and they may be either redundant or have other roles.

Gallon JR (1992) New Phyt. 122, 571-609.

ATPase IN HOST CELLS AND BACTEROIDS IN LEGUME NODULES

JING YUXIANG and SHAN XUEQIN
Institute of Botany, Chinese Academy of Sciences, Beijing
100044, China

The study on location of ATP-ase in root nodules of Astragalus sinicus and Sesbania cannabina showed that the particles of lead phosphate precipitates resulting from the reaction of ATP hydrolytic enzyme are distributed in cell wall, plasmolemma, cytoplasm and peribacteroid membranes (PBM) etc. of host cells in the nodules. Owing to the need of photosynthates provided for nitrogen fixation of the root nodules, the ATP-hydrolase plays an important role in the transportation and absorption of substances.

The active reaction of ATP-hydrolase varies with their developments. The numbers of bacteroids having ATP-hydrolase in the young root nodules are significantly less than those in the senescent nodules. Possibly, this active reaction is related with the physiological function of nitrogen fixation in the bacteroids at different development stages.

The nitrogenase activity of the root nodules from Sesbania cannabina plants treated with $Na_2S_2O_4$,DTT and tryptin was increased by 108-114%, 106-117% and 103-119%, respectively. EM observation showed that the density of ATP-hydrolase as the marker of lead phosphate particles which were distributed on the PBM was much significant than that of the control, but the bacteroids in PBM did not have ATP-hydrolase particles present. Dark treatment of the same age plants accelerated the nodule senescence and the ATP-hydrolase particles most densitied on PBM of the nodules, meanwhile, the dense ATP-hydrolase particles also appeared in a number of the degenerative bacteroids.This confirms the conformation change of ATP-ase in the bacteroids from ATP synthetase to ATP-hydrolase and its relation to nitrogen fixation with senescence of the nodules.

UPTAKE HYDROGENASE IN *BRADYRHIZOBIUM SP. (LUPINUS)*

LISOVA, N.
Research Institute of Agriculture and Cattle-Breeding of the West Region, UAAN, Lviv-Obroshyn, Ukraine

The reduction of dinitrogen to ammonia by nitrogenase is known to be connected with H_2 formation. This causes a loss of energy and reduction equivalent and deacreases the ammonia output. However, a number of N_2-fixing microorganisms are able to recycle the evolved H_2 via an uptake hydrogenase (Hup^+ - strains). Until recently, the uptake hydrogenase was studied in the strains of *Rhizobium leguminosarum* and other fast-growing rhizobia. In 1989 the Hup^+ strains of *Bradyrhizobium sp.* (*Lupinus*) were reported.

We studied 19 strains of *Bradyrhizobiun sp. (Lupinus)* of various activity under the symbiotic conditions. Four among them (363a, 357a, 67, 3045) being distinguished by the extremely low electron allocation coefficient for proton reduction were assigned to the Hup^+ - type. The advantage of the Hup^+ - strains over the Hup^- - strains of high activity in the symbiotic effectiveness was very variable over the years of the vegetation season, being related to the total solar radiation. The relationship was due to comparatively lower response of the Hup^+ - symbiosis effectiveness (the phytosymbiont productivity).

The inversely relation between electron allocation coefficient for proton reduction and the level of Hup - symbiotic effectiveness was established for lupine.

The varieties specificity of an expression of the hup-genes for the studied strains was found. Plant green biomass, nitrogen content in grain and nitrogenase activity were found to be higher in lupine inoculated with Hup^+ - strains. Hup- character of the strains was not found to influence the nodule mass. Results of the investigation of *Bradyrhizobiun sp. (Lupinus)* strains suggest that Hup^+ - phenotype resulted in the increased accumulation of nitrogen in grain.

NITROGEN FIXATION AND HYDROGENASE IN *FRANKIA* ISOLATED FROM *CASUARINA*.

MATTSSON U., SELLSTEDT A.
Dept of Plant Physiology, University of Umeå, S-901 87 Umeå, Sweden

Frankia differentiates into three different cell types; hyphae, vesicle and sporangia. In free-living state, *Frankia* can grow on a defined nutrient solution and when nitrogen is withdrawn from the media, oxygen limiting vesicles are formed to protect the oxygen sensitive nitrogenase. Vesicles are also found in most of the *Frankia* symbioses. However, in nodules of *Casuarina* sp. vesicles have, so far, never been found. Immunogold localization revealed that nitrogenase and uptake hydrogenase was located in the hyphae of the *Casuarina-Frankia* symbiosis (Sellstedt A, Mattsson U, 1994). Although, free-living *Frankia* isolated from *Casuarina* sp do form vesicles when grown in nitrogen free media.

When free-living *Frankia* KB5, isolated from *Casuarina equisetifolia*, grown in a defined media with nitrogen, is transferred to medium without nitrogen, vesicles are formed after about three days. However, the nitrogenase activity, measured as ARA, does not reach the optimum value until after eight days in nitrogen free media. Uptake hydrogenase activity, measured with an hydrogen electrode, shows a similar pattern as nitrogenase on a time scale, but reaches the optimum value one day later.

This indicate that uptake hydrogenase activity is stimulated by the dihydrogen procuced concomitantly via nitrogenase as nitrogen is fixed. By using antibodies against uptake hydrogenase holoenzyme purified from *Alcaligenes latus* (Pinkwart M et al, 1993, Doyle CM, Arp DJ, 1987) in a western blot analysis revealed that a polypeptide at 60kD corresponding to the large subunit of uptake hydrogenase was present in *Frankia* KB5 after 5 days of growth in nitrogen free media.

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THE *GUNNERA-NOSTOC* SYMBIOSIS

RASMUSSEN, U., JOHANSSON, C. & BERGMAN, B.
Dept. of Botany, Stockholm Univ., S-10691 Stockholm, Sweden

The N_2-fixing cyanobacterium *Nostoc* has a wide symbiotic host range, but it enters into symbiosis with only one angiosperm, *Gunnera*. Specialised glands formed on the *Gunnera* stem independent of the presence of cyanobacteria are infected by motile *Nostoc* hormogonia. Subsequently delimited areas of intracellularly infected cells are formed within the *Gunnera* stem.

Little is known about the molecular events underlying establishment of this symbiosis. Among several *Gunnera* exudates and extracts tested, only the mucilage induced hormogonium differentiation. The inducing factor was a protein with M_w<12kDa. Hormogonium induction was observed in both compatible and incompatible strains. No chemotactic response of the hormogonia towards the *Gunnera* mucilage was detected. However, the mucilage had a growth-stimulating effect on compatible strains. The mucilage also caused a rapid synthesis of at least two specific proteins (40 and 65 kDa) in compatible strains only. This appears to be a symbiosis-specific response, which may have a vital function during early phases of establishment. Hence, *Gunnera* mucilage carries chemical signals involved in communication with *Nostoc*[1].

Analyses of non- and carboxyl reduced mucilage gave a composition of arabinose, galactose and glucose in relative molar proportions of 1.00:0.25:0.13, with glucose probably as a uronic acid. Characterisation using the specific monoclonal antibody MAC 207[2] directed against arabinogalactan proteins (AGPs) gave a strong reaction on dot-blot analysis. AGPs are implicated in differentiation of plant cells and tissues, and might be responsible for the effects seen on *Nostoc*.

Heterologous hybridisation of various rhizobial and agrobacterial genes was performed with *Nostoc* DNA from compatible and incompatible strains. *Nostoc* showed homology to several of the genes, among which the *pic*A gene, which is induced by acidic polysaccharides in *A. tumefaciens*, showed hybridisation only to compatible *Nostoc* strains. This may be of importance during establishment of the *Gunnera-Nostoc* symbiosis.

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THE EFFECT OF PROTON THE PUMPING ACTIVITY OF THE PERIBACTEROID MEMBRANE ATPASE OF PEA NODULES ON THE NITROGENASE ACTIVITY

SZAFRAN[1] M.M. AND HAAKER[2] H.
[1]Faculty of Commodity Science, Academy of Economics, Poznañ, Poland,
[2]Department of Biochemistry, Agricultural University, Wageningen, The Netherlands

In the root nodules of legumes the nitrogen fixing bacteroids are separated from the cytoplasm of the plant cell by a plant-derived membrane called the peribacteroid membrane. This membrane provide the plant with means for regulating nutrient exchange with the microsymbiont and potentially a way to control nitrogen fixation. Kinetic properties of the peribacteroid membrane such as the apparent K_m at 0.52± 0.06 mM ATP, moderate salts stimulation, competitively vanadate inhibition and absence of NO_3^- inhibition could classify peribacteroid membrane ATPase as H^+-pumping plasma membrane. The ATPase activity associated with this membrane preparation was characterized and its electrogenic properties were determined. The fluorescence emission of ACMA was quenched after adding MgATP what indicated that the ATPase generates pH gradient inside acidic across the peribacteroid membrane. The observed quenching of fluorescence was reversed by the protonophore TTFB, by the inhibitor of the ATPase activity vanadate and by NH_4Cl. NH_4^+ dissipates a ΔpH by diffusion as NH_3 through membranes and reacting inside with H^+. The generation of membrane potential (positive inside) was detecting by a red shift of the absorbance of Oxanol VI at 630 nm relative to 608 nm after adding MgATP. The addition of valinomycin, the protonophore TTFB and NO_3^-, all collapsing the membrane potential, reversed the spectral shift. NH_4Cl did not collapse the Oxanol VI response what indicates that NH_4^+ only dissipates the ΔpH but not the $\Delta\Psi$. The reversibility of the ATPase was determined by a light-dependent ATP formation from ADP and P_i of peribacteroid membrane vesicles fused with the purple membrane from Halobacterium halobium and phospholipid. At pH 7.4 and 6.5 the addition of MgATP to the symbiosomes strongly inhibited nitrogenase activity. Inhibition of the peribacteroid membrane ATPase by vanadate or the dissipation of the pH gradient by NH_4Cl reversed the inhibition by MgATP. At pH 8.4 where the pH of the medium inhibits the nitrogenase activity, one expects a stimulation of the nitrogenase activity when the peribacteroid membrane ATPase is active. In nodules the peribacteroid membrane ATPase activity might inhibit nitrogenase activity. The air space in the root nodules were filed with a vanadate solution by evacuating the root nodules in this solution. The stimulation effect of vanadate could be explained as an inhibition of the peribacteroid membrane ATPase and thus by increasing the pH of the peribacteroid space to a more optimal pH for nitrogenase fixation by the bacteroids.

EFFECT OF ELEVATED ATMOSPHERIC CO_2 ON PERFORMANCE OF SYMBIOTIC NITROGEN FIXATION IN WHITE CLOVER IN THE FIELD (SWISS FACE EXPERIMENT) AND POSSIBLE ECOLOGICAL IMPLICATIONS.

ZANETTI S., HARTWIG U.A., HEBEISEN T., LÜSCHER A., HENDREY G.R., BLUM H. and NÖSBERGER J. Institute of Plant Sciences, Federal Institute of Technology, 8092 Zürich, Switzerland.

The effect of an elevated atmospheric CO_2 concentration (60 Pa vs. ambient) on symbiotic N_2-fixation (^{15}N-isotope dilution method, *Lolium perenne* L. as reference) in white clover (*Trifolium repens* L.) was investigated using the Free Air Carbon-dioxide Enrichment (FACE) technology. The experiment was set up as a model ecosystem (14 or 56 g N m^{-2} a^{-1}, 4 or 8 cuts per year) on fertile soil near Zürich. Under elevated CO_2, above-ground biomass production in white clover monocultures increased by 20% in the first two experimental seasons (Hebeisen *et al.*, unpublished data). Performance of symbiotic N_2-fixation (percentage of nitrogen derived from symbiosis) increased by 15%. This CO_2-effect was evident in both nitrogen applications and through both harvest regimes during the entire seasons although more prominent during summer. Total yield of fixed nitrogen increased by 35%. The entire increase in above-ground nitrogen yield under elevated CO_2 could be attributed to the increased N_2-fixation; no additional nitrogen was assimilated from the soil. This is in contrast to a growth chamber experiment (plants were growing in silica sand with nutrient solution), where increased biomass production under elevated CO_2 was not associated with increased percentage of nitrogen from symbiosis. In the field experiment, it is suggested that due to the higher C-sequestration into the entire ecosystem under elevated CO_2, the total N sink of the ecosystem increased, leading to more vigurous competition for mineral N and thus the availability of mineral N decreased. Such a concept would be consistent with the weak above-ground biomass response of the associated *Lolium perenne* (+ 5%), the dramatically increased root biomass response and the decreased nitrogen yield in the above-ground biomass. Thus, the observed increased symbiotic N_2-fixation under elevated CO_2 reflected not only an increased N-demand of white clover but also an increased N-sink of the whole ecosystem. In summary, under conditions of a raising atmospheric CO_2-concentration, symbiotic N_2-fixation could be viewed as a process to maintain the C:N balance in the ecosystem and thus being one factor allowing additional C-sequestration from elevated CO_2.

SECTION 8

ECOLOGY AND AGRICULTURAL APPLICATIONS OF NITROGEN-FIXING SYSTEMS (SPONSORED BY OECD)

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ECOLOGY AND AGRICULTURAL APPLICATIONS OF NITROGEN-FIXING SYSTEMS: OVERVIEW

HARDY, R. W. F. AND EAGLESHAM, A. R. J.
Boyce Thompson Institute for Plant Research, Inc., Ithaca, NY 14853, USA

Global agricultural consumption of synthetic nitrogen fertilizer increased 27-fold between 1950 and 1990 to approximately 80 million metric tons per year, about 50% of the total amount of nitrogen fixed by biological and physical processes. The impact of industrial fixation on the global nitrogen cycle is yet to be assessed, but it would seem prudent to minimize further perturbation.

At best, only about half of the nitrogen applied to crops actually contributes to yield; the rest is lost to the environment. Probably the most significant adverse consequence of modern production agriculture is nitrate, not pesticide, contamination of groundwater and soil. Denitrification of nitrate produces about 90% nitrogen gas and 10% nitrous oxide, a greenhouse gas with energy reflectivity 180 times that of carbon dioxide. The increase in nitrous oxide content of the troposphere during the 1980s correlates more strongly with increased use of fertilizer nitrogen than with the increase in combustion of fossil fuel. The global budget for nitrous oxide is out of balance, with sources exceeding sinks by 30-40%; its concentration in the atmosphere is increasing by 0.25% per year (Vitousek, Matson, 1993). Furthermore, the industrial production process constitutes a major consumption in fossil energy and a huge and growing economic cost of more than US$20 billion per year.

The greatly expanded investment in nitrogen-fixation research that occurred in the 1970s as a consequence of the energy crisis increased our fundamental knowledge of asymbiotic and symbiotic systems but has not yet produced necessary practical applications. A recent analysis of these applications tabulated them in terms of potential benefit and risk of successful development and use (Hardy, 1993). In Symposium 7, selected lines of research are described that offer potential to alter traits of bacteria and crop plants to increase BNF and decrease reliance on synthetic fertilizer. There is need to better understand key factors that govern the microbial ecology of the rhizosphere and influence saprophytic competence, competition for nodulation by rhizobia, and both beneficial and pathogenic microbiota. Recent results suggest that microbial inoculants may improve crop yields without significant perturbation of the indigenous rhizosphere microflora (O'Gara, Nuti, 1995). In Israel, beneficial effects on growth and yield of many crops have resulted from inoculation with *Azospirillum*; a 60-70% success rate and statistically significant increases in yield of 5-30% have been obtained (Okon, 1995). Growth promotion accrues from improved development and function of roots; therefore, research on rhizosphere ecology and on

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physiological and genetic aspects of bacterium-root interactions may lead to higher frequency and level of positive response to inoculation with *Azospirillum*.

Quantitative trait loci have been identified for significant, albeit modest, inputs of rhizosphere-associated nitrogen fixation in paddy rice, demonstrating that genetic factors regulate interactions with diazotrophs in the rhizosphere (Reddy, Ladha, 1995). However, greater BNF may occur in rice that is genetically manipulated to maintain more-intimate associations with diazotrophs, either as root nodules or as stem- and leaf-borne endophytes. The highest estimates of plant-associated nonsymbiotic nitrogen fixation have been obtained with certain cultivars of irrigated sugarcane, and several species of endophytic diazotrophic bacteria have been isolated in high numbers from the leaves, stems and roots of field-grown plants. *Acetobacter diazotrophicus*, one of the endophytes, is believed to be the chief contributor of fixed nitrogen (Boddey et al., 1995). Various manipulations are being examined to inoculate uninfected sugarcane plants to maximize endophytic BNF.

Progress in improving inoculant formulations ensures that high numbers of bacteria are applied to the soil with separation from chemically treated seed and convenience of use for growers (Smith, 1995). Pretreatment with compounds that elicit the expression of nodulation genes in cultured rhizobia may improve the competitive ability of strains with high nitrogen-fixing capability (Thomas et al., 1995). Broad host-range rhizobia, strains of *Rhizobium tropici*, that nodulate common bean (*Phaseolus vulgaris*) have been found to fix nitrogen efficiently even at high temperatures and low pH. Research is in progress to determine if broad host-range correlates with saprophytic competence, rhizosphere-colonizing ability and competitiveness for nodulation (Vlassak et al., 1995).

Continuing and enhanced R&D in BNF is more justified in the 1990s than in the 1970s; it is impossible to conceive sustainable agriculture without replacement of synthetic fertilizer nitrogen with biologically fixed nitrogen. A U.S. National Research Council Committee report in 1994 concluded: "A high priority for increased investment in BNF is justified by the opportunities to reduce or replace the growing cost of fertilizer nitrogen, currently more than $20 billion per year, to minimize the multifaceted local and global environmental damage that it causes and to sustain the food needs of an expanding world population. Expansion of our knowledge and development of economic applications and management systems for developed and developing countries should be pursued." (NRC, 1994). There must be an appropriate balance of funding of fundamental and applied BNF research and a coupling of the two activities.

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ECOLOGY AND AGRICULTURAL APPLICATIONS OF NITROGEN-FIXING SYSTEMS: CROPS AND SCIENCES INVOLVED

WERNER D.
Philipps-Universität, FB Biologie, Karl-von-Frisch-Straße, D-35032
Marburg, Germany

Grain and forage legumes are still by far the most important harvesters of N_2 used for agricultural practices. Grain legumes are planted annually worldwide on almost 1.5 million km^2 of land, resulting in an annual production of about 200 million tons with about 10 million tons of nitrogen in the seeds. Approximately half of this area is planted with soybeans and 20% with various subtropic legumes such as *Cicer*, *Cajanus* or lentils. Peanuts grow on about 10% and the rest is divided among *Phaseolus* sp., *Pisum* sp. and *Vicia faba*. The area covered by forage legumes is even much larger due to the grassland area of about 30 million km^2 on the five continents, mixed with legumes up to 50% of the area. The most important genera in temperate, tropical and subtropical areas are *Trifolium*, *Lotus*, *Medicago*, *Stylosanthes*, *Macroptilium* and *Mimosa* sp. (Werner, 1992). The third group of large area covering nitrogen-fixing plants are N_2-fixing trees from the genera *Acacia*, *Alnus*, *Erythrina*, *Leucaena*, *Robinia* and *Casuarina* (Brewbaker, 1990). A large potential for increased utilisation of N_2 fixation by agricultural crops rests in the established large differences in the percentages of N derive from air from the total nitrogen assimilated by legumes. The figures from a coordinated research programme FAO/IAEA are 40% for common bean, 50% for soybean and cowpea, 65% for chickpea, 75% for faba beans, 80% for alfafa and clover and almost 95% for lupins (Hardarson, 1993). All forage legume species apparently fix a significantly higher percentage of N_2 from air than all the grain legumes. More studies on the sink-source relationship and competition between leaves and seeds within grains and forage legumes could be a research priority in the future. With *Phaseolus vulgaris* improved breeding lines with a high percentage of N_2 fixation have already been produced (Bliss, 1993). The well-established effect of *Rhizobium* and *Bradyrhizobium* strains on the N_2 fixation efficiency also deserves further molecular and applied studies and is well underway in several laboratories, represented in this symposium and this congress.

In the OECD cooperative research programme "Biological Resource Management for Sustainable Agricultural Systems" started in 1995 for another five years period (Dr. Muriel Dunier as Head of Project), there are two themes related to the topics of this symposium: theme 1 'Safe exploitation of micro-organisms in plant/soil systems' with J. Lynch as scientific coordinator and theme 3 'Utilisation and ecology of new organisms" with H. Hokkanen in the same position. In these two themes the following

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topics have been identified as important for future research relevant to our symposium.

- Methods for molecular ecology
- Production and delivery of microbial inocula
- Reduction of the load of chemical pesticides, fertilisers and organic wastes on the soil ecosystem
- Introduction of new organisms and transgenic crop plants including non-food plants
- Population biology of new organisms in agricultural systems

In this respect we must be very careful to avoid overpromising new ideas. A little new outgrowth from a root system is by far not the start of something similar to a highly organized legume nodule. Invading root bacteria, even if they reach 10^8 cells per g of root tissue have to be compared with the more than 10^{11} bacteroids inhabiting 1g of effective legume nodules. Nevertheless non endosymbiotic systems deserve also continuing efforts and their efficiency must be compared with the most effective symbioses.

Competition for nodulation and N_2 fixation efficiency of N_2-fixing microsymbionts as *Rhizobium*, *Bradyrhizobium*, *Azorhizobium* and *Frankia* must also be connected to progress in our understanding of plant growth-promoting rhizobacteria in general and their effects against various plant pathogens as bacteria, fungi and nematodes (Mahaffee, Kloepper, 1994). The physical, chemical and molecular understanding of these interactions need, on the other side, a better understanding of soil structure/soil biota interrelationships in general (Brussaard, Kooistra, 1993).

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RECENT DEVELOPMENTS IN BIOTECHNOLOGY AND THE POTENTIAL IMPACT ON NITROGEN FIXATION IN THE FIELD.

O'GARA F.(1), MOENNE-LOCCOZ Y., DOWLING D.N. and
NUTI M.P.(2)
(1) Microbiology Department, University College Cork, Cork, Ireland
(2) Dipartimento di Biotecnologie Agrarie, University di Padova, Padova, Italy.

Abstract

Genetic modification offers considerable potential to alter specific cellular traits for improved nitrogen fixation performances. However, to achieve the true potential from nitrogen fixing microorganisms under normal agricultural practice it is necessary to have a better understanding of the factors governing the microbial ecology of the rhizosphere. Relevant factors include saprophytic competence of the introduced strain, competition for nodulation (in the case of a symbiosis) and the influence of both beneficial (mycorrhizae) and detrimental (plant pathogens) members of the rhizosphere microbiota. The role of key microbial components in maintaining the nitrogen fixation process for plant productivity needs to be better understood. Molecular tools are now available to probe and modify these interactions, and emerging molecular microbial ecology approaches are being applied in the rhizosphere. In a European Union multidisciplinary research initiative, a number of laboratories are collaborating in the framework of the IMPACT project to investigate the performance and interactions of wild type and genetically modified microbial inoculants.

The experiments include inoculation of N_2-fixers with biocontrol strains and evaluating the effect of inoculants on the indigenous microbiota including mycorrhizal fungi. Results will be discussed suggesting that the microbial inoculants have the potential to improve crop yields while causing minimum perturbation to the indigenous rhizosphere microbiota.

Introduction

Efficient nitrogen fixation in the field will contribute to improving the production of good legume yields. The legume yield can be influenced by a number of factors including the genotype of the plant, the nitrogen fixing inoculant available and the environmental and ecological conditions in which the plants are growing.

Biotechnology can contribute significantly to the multidisciplinary approach needed to improve nitrogen fixation in the field. Recent advances in biotechnology have lead to the development of transgenic plants and microorganisms. In the context of transgenic plants, new products are being developed with altered phenotypes such as herbicide resistance, pest resistance and metabolic alterations

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influencing traits such as ripening and flavour characteristics. In addition, the concept of 'molecular farming' where transgenic plants are being exploited to produce value added products such as pharmaceuticals is gaining momentum. However in the case of legume plants, there are still some technical problems to be overcomed in the generation of commercially useful transgenic legumes (see Gresshoff et al., and Verma et al., in this volume).

Nevertheless the application of innovative biotechnology to legumes is continuing to improve and contribute to our knowledge based on key genes and their role in the process of nodule formation and function.

The Role of Innovative Biotechnology in Improving Nitrogen Fixation in the Field.

In the context of the application of biotechnology to microorganisms, much success has been achieved in exploiting and modifying microorganisms as cell factories. Cellular traits have been modified that include enzyme, antibiotic and amino acid production. In addition microorganisms have been exploited for the production of novel pharmaceutical or health care products such as hormones, and antiviral agents. The success of these initatives has been facilitated by the fact that the producing microorganisms are cultivated in a contained environment in fermenters in which the environment conditions can be chosen and controlled.

In contrast to this industrial situation the genetic modification and use of microbial seed inoculants in agriculture is more complex. Microbial inoculants, when delivered into natural environments such as soil and the rhizosphere must operate and function under the prevailing environmental and ecological conditions into which they have been introduced. This is a common factor when dealing with microbial inoculants whether they are designed and used as biopesticides, biofertilizers, phytostimulators or used in soil bioremediation.

Microbial inoculants can be improved by genetic engineering procedures under laboratory based conditions. However for application under field conditions consideration must be given to understanding the interaction between microorganisms and the nature of the signals they respond to in natural environments.

Molecular biology of the symbiosis: improvements to nitrogen fixation.

Molecular techniques developed in biotechnology have played a major role in our undertanding of the symbiosis and nitrogen fixation (See chapters in Sections 4-7 in this volume). The discovery of the lipo-chitin-oligosaccharide 'Nod factor' signal molecules and the understanding of the factors involved in the cascade system of regulation of nitrogen fixation in free living and bacteroid forms of microbial inoculants is providing the necessary basis to develop improved inoculants. For example genetic manipulations involving the nitrogen fixation regulatory gene *nifA* and the genes involved in dicarboxylate transport (*dctABD*) in *Rhizobium meliloti* have been developed (Cannon et al., 1988; Ronson et al., 1990). The alfalfa yield response to inoculation with recombinant strains of *R. meliloti* with an extra copy of *dctABC* and/or modified *nifA* expression was evaluated under agricultural conditions (Bosworth et al., 1994). The rational for the investigation was based on the regulatory role that *nifA* plays in the *nif* genes and that a supply of dicarboxylates are provided by the plant to the bacteroids in the nodule. Recombinant strain RMBPC-2 which had an additional copy, of both *nifA* and *dctABD* , increased alfalfa biomass by 12.9% compared to the yield with the wild type strain RMBPC where soil nitrogen and organic matter were low (Bosworth et al., 1994). At three other sites either resident rhizobial populations or soil nitrogen concentrations were high and at these sites none of the recombinant strains affected yield. Obviously repetition of similar field trials would be necessary to determine if this pattern is reproducible. However, this approach does point out that it is important to understand the nature of environmental and ecological factors that influence the symbiotic system. Innovative biotechnology can provide the tools to do this and in recent years molecular microbial ecology has been emerging as a key approach to help understand symbiotic nitrogen fixation in the field.

Molecular Microbial Ecology

Molecular microbial ecology deals with the application of molecular biology to microbial ecology. It facilitates the detection and identification of microorganisms in the environment. It enables new approaches for the exploration and exploitation of microbial diversity to be assessed. Furthermore, it provides a sensitive framework in which impact analysis of strains and/or their metabolites on the environment can be addressed.

When considering the ecological factors affecting nodulation and nitrogen fixation in the field it is clear that plant factors such as root exudates and the indigenous microbial community are some of the important parameters. For nitrogen fixation in the field, rhizobia must function as part of a complex microbial community. The application of molecular microbial ecology can provide the basis to understand the factors and interactions that influence rhizobia in the field and in the rhizosphere. These interactions can cover the impact on mycorrhizal fungi, biocontrol strains and other soil microbiota, fungal - bacterial and plant -bacterial interactions.

Providing an integrated approach for nitrogen fixation in the field is becoming more important. Global trends are now demanding more enviromental friendly agriculture. The inputs for this will rely more heavily on exploiting biofertilizers such as nitrogen fixing bacteria and mycorrhizal fungi, and biopesticides such as biocontrol strains. The agricultural outputs emerging from this approach will include improved food quality, sustainable food production and environmental protection.

Impact and compatability of biological inoculants The Biotechnology programme of the European Union has supported these new global initiatives and is funding a research network called IMPACT to investigate the Interactions between Microbial inoculant and resident Populations in the rhizosphere of Agronomically important Crops in Typical Soils (Boesten et al, 1993). The objective of the IMPACT project which includes seventeen centres of excellence and industries in different EU member states is to assess the ecological impact of wild type or genetically modified inoculants on key biological components of the rhizosphere .

In keeping with the demand for more integrated and sustainable approaches for food production the IMPACT project is evaluating the combined use of rhizobial biofertilizer inoculants and biocontrol strains for phytopathogen control under field conditions. It is essential that both biocontrol agents and biofertilizers are compatible for use under field situations.

The biocontrol strain *P. fluorescens* F113 produces a range of anti-microbial substances (see Fig 1) including the metabolite 2,4-diacetylphloroglucinol (DAPG), which is a potent fungal inhibitor. It is important that such potential microbial biopesticides be tested not only for their impact on the resident microbiota but also for their possible interactions with microbial biofertilizers e.g. rhizobia and mycorrhizal fungi.

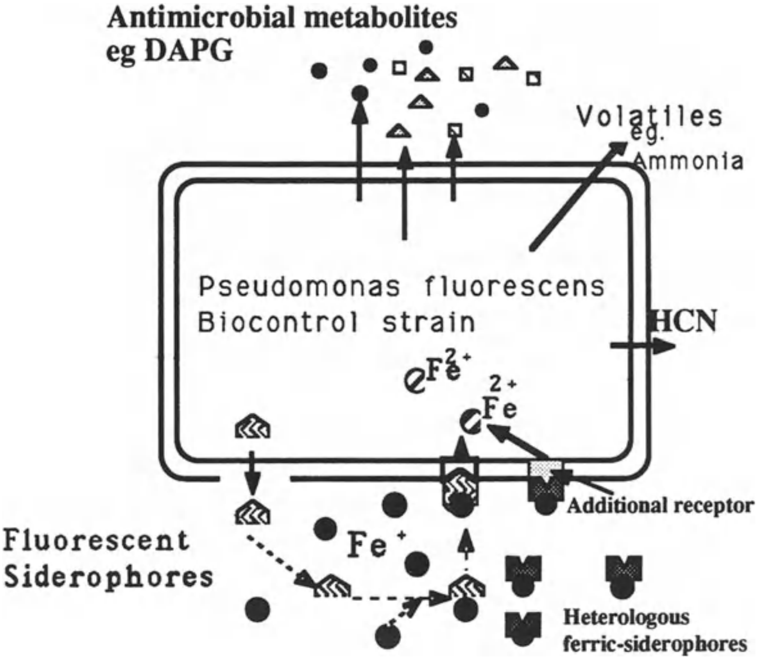
Antimicrobial metabolites
eg DAPG
Volatiles
eg.
Ammonia
Pseudomonas fluorescens
Biocontrol strain
HCN
Fe2+
Fe 2+
Additional receptor
Fluorescent
Siderophores
Fe+
Heterologous
ferric-siderophores

Fig. 1. Metabolites implicated in biocontrol by *Pseudomonas*

In a field experiment initiated in Northern Italy in 1994, soybean was co-inoculated with a genetically modified *P. fluorescens* anti-microbial metabolite producing biocontrol strain and a commercial *Bradyrhizobium japonicum* inoculum. Interestingly, although the genetically modified (GM) *P. fluorescens* inhibited the *B. japonicum* inoculant *in vitro*, a significantly stimulatory effect on *B. japonicum* nodulation was observed under field conditions. However, the improved nodulation score did not translate into differences of seed yield at harvest (Corich et al, 1995).

In a similar experiment, with respect to inoculant interactions with the beneficial vesicular arbuscular mycorrhizae (VAM), field data indicated that pea roots evaluated for VAM entry points were unaffected by the inoculation with either wildtype of genetically modified rhizobia (Corich et al, 1995).

In order to increase our familiarity with the safe use of genetically modified microorganisms (GMMs) it is useful to compare and evaluate the effect of GMMs on crops and the resident microbiota compared to wildtype strains. Field work to date indicates that introduced genetically modified bacteria become established in the rhizosphere of plants similar to the isogenic WT strains (Corich et al., 1995). Apart from the introduction of *P. fluorescens* F113 which can be established at levels of 20 -50% of the total fluorescent population in the rhizosphere, introduced rhizobial strains showed a decline in the culturable population.

An analysis of the rate of carbon dioxide and nitrous oxide (N20) emissions in soil inoculated with genetically modified and non-modified *Rhizobium leguminosarum* bv. *viciae* has indicated the following :

1. Soil without plants had a lower respiration rate than soil with inoculated plants whether or not they were inoculated with GM or wildtype rhizobia (Corich et al., 1995).

2. Similarily with N20 emissions, significant differences could not be detected between soil inoculated with GM and non-modified strains. However, N20 emission from soil without plants was significantly lower compared to soil with plants (Corich et al., 1995).

3. Vesicular-arbuscular mycorrhizae of pea roots was monitored (for 2 months after sowing) and was found to be affected by the type of bacterial inoculum GM or wildtype *Rhizobium* (Corich et al., 1995).

These results indicate that the presence of plants (and rhizospheres) has a major impact on carbon mineralisation in soil. However, the data suggests that the behaviour of isogenic wildtype/modified strains is essentially similar during these field experiments.

Impact of biological control agents on mycorrhizae

Within the IMPACT project the interactions between *Pseudomonas* biocontrol strains and VAM fungi are also being evaluated, initially under *in vitro* conditions. Fluorescent pseudomonads can colonise VAM hyphae (Bianciotto et al 1994) and the biocontrol strain F113 has been shown to stimulate germination of the spore and subsequent mycelium development compared to uninoculated controls (Vidal et al, 1994). These preliminary results encourage the view that compatibility between the important VAM-plant interaction and inoculation with rhizobial and/or biocontrol agents will be feasible and in some cases synergistic.

Conclusions

In conclusion biotechnology, as applied to *(Brady)Rhizobium* ecology is providing new information and applications to improve inoculant use. It is clear that it will be possible to achieve integration and sustainable agricultural practices by combining *Rhizobium* as biofertilizers + biocontrol inoculant + mycorrhizal fungi to produce compatible multipurpose inoculants. Linking these approaches for field use will provide an attractive package for agro-industry / farmers and growers that represents a substantial benefit from agricultural biotechnology.

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CAN SYMBIOTIC NITROGEN FIXATION BE EXTENDED TO RICE?

REDDY, P. M. AND LADHA, J. K.
Soil Microbiology Section, Soil and Water Sciences Division, International
Rice Research Institute, Philippines

INTRODUCTION

Rice is the principle food for more than half of humankind. Over the past two and a half decades, farmers have become increasingly dependent on chemical sources of N for obtaining higher yields of rice to meet the demands of enlarging population. With the gradual depletion of petroleum reserves, increased environmental concerns as well as production costs and low use efficiency of chemical N fertilizer, now is the time for supplementary and/or alternate sources of N to be seriously considered for rice, besides exploring ways for efficient use of soil and fertilizer N, in order to meet increasing demands in coming years. It is in this context that biological nitrogen fixation (BNF)-derived N assumes importance in the lowland soils that provide about 86% of the world's rice.

Among the conventional BNF systems of rice, the free-living/associative diazotrophs have low to moderate potential to supply N to rice because N_2 fixed outside the plant is subjected to loss. Green-manure crops like *Azolla* and *Sesbania* have high N supply potential to support higher grain yields of rice, but farmers are reluctant to use them as there is no economic advantage over N fertilizer (Ladha, Reddy 1995). If a BNF system could be assembled in rice plant itself, it can enhance N supply potential as fixed N would be available directly to the plant with little or no loss, besides ensuring no additional economic burden on farmers. To address this challenge, we assess the possibilities for developing novel and unconventional symbiotic BNF systems for sustainable rice production.

DEVELOPMENT OF RICE-ENDOPHYTIC DIAZOTROPH ASSOCIATIONS

Diazotrophs like *Acetobacter diazotrophicus* and *Herbaspirillum* spp. grow endophytically in the stems and leaves of sugar cane. There are evidences to show that *A. diazotrophicus* is the main contributor of endophytic BNF, which according to N-balance studies was found to be as high as 150 kg N ha^{-1} $year^{-1}$ in this crop (Boddey et al. 1995). Another N_2-fixing endophyte which is of considerable interest is *Azoarcus*. This diazotroph inhabits the roots of Kallar grass (*Leptochloa fusca*) which yields 20 - 40 t of hay ha^{-1} $year^{-1}$ without the addition of any N fertilizer in saline-sodic, alkaline soils having low fertility (Sandhu et al. 1981). Inoculation experiments with *Herbaspirillum* spp. in non-sterilized soil under greenhouse conditions have shown that these endophytic

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diazotrophs can be readily introduced into rice plant by application of the bacterial cultures on seeds prior to germination (Olivares et al. 1993). Infection occurs through roots as well as via stomata and the diazotrophs are translocated through the xylem to all parts of the plant. *Herbaspirillum* spp. when inoculated into rice seedlings growing in N-free Hoagland solution, containing ^{15}N-labeled N, showed ^{15}N dilution amounting to a 40% increase in total plant N (Dobereiner et al. 1993). Dobereiner et al. (1993) speculated that the endophytic diazotrophs in certain rice genotypes may, in fact, be responsible for the substantial contributions of BNF to rice reported earlier (App et al. 1984). Our recent studies have shown that rice, indeed, harbours a wide range of endophytic diazotrophs. Presently, efforts are underway to evaluate the extent of colonization and N_2 fixation in rice by these endophytes.

Once an effective endophytic diazotroph is identified, it is important to have an efficient method of inoculation to enhance the chances of the inoculated endophyte to selectively colonize the rice plant, because the inoculated microbes are usually outcompeted by the indigenous microflora in the rhizosphere of crop plants. In order to ameliorate this problem, de Bruijn and his associates are examining the possibility of creating "biased rhizosphere", a novel procedure to selectively encourage the growth of introduced microorganism in rhizosphere (Rossbach et al. 1995). In this system, the nutritional compound expressed by the transgenes in a transgenic plant and secreted into soil environment can only be utilized by the bacteria possessing genes for catabolism of this compound. If such catabolism genes are incorporated into the beneficial bacteria and inoculated into the rhizospheric zone of the transgenic plant, the chances for these bactria to be able to survive, in the face of the competition offered by indigenous microflora, will be better. This is because the novel compound secreted by the transgenic plant can only be utilized by these microbes which are equiped with the ability to metabolize this compound as carbon source. In this way the introduced microbe need not compete with local strains for common carbon sources for their survival in new environment. Though this novel approach seems to be achievable, one has to consider that many other factors (both biotic and abiotic stresses), besides the availability of carbon source, regulate survival and establishment of microorganisms in new environments.

The entry of *A. diazotrophicus* into sugar cane/sweet sorghum roots is facilitated by VA mycorrhizae (Paula et al. 1991). The diazotroph/ mycorrhiza interaction is another area which needs attention for the development of effective endophyte-delivery systems for rice.

DEVELOPMENT OF RICE-RHIZOBIA SYMBIOSES

Currently there is much interest in determining whether rhizobia would be able to nodulate monocots such as rice, and carry out N_2 fixation. In fact, the possibility of extending the host range of rhizobia to non-legumes was encouraged by the discoveries that *Parasponia* forms nodules with *Rhizobium* (Trinick, 1973), and that *Rhizobium parasponium* RP501 and *Bradyrhizobium* CP283 induce nodulation in oilseed rape (Cocking et al. 1990, 1992).

A variety of investigations aimed at understanding legume-nodule development form a basis for devising rational strategies to extend host range of N_2-fixing symbioses to nonlegume crops. Soil bacteria of the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* (collectively referred to as rhizobia) interact with leguminous plants to form N_2-fixing nodules through a process which begins with the secretion of flavonoids from roots, and consequent flavonoid-triggered *nod* gene expression in microsymbiont leading to the production of Nod factors. Nod factors induce root hair deformation and promote the processes involved in initiation of infection thread and cortical cell division during the early steps of the nodulation in legumes (Ardourel et al. 1994). Nevertheless, it is yet to be determined whether the morphogenetic changes related to nodule development are under the direct control of Nod factors or regulated through Nod factor-mediated alterations in the cytokinin-to-auxin ratio in root cortical cells (Cooper, Long 1994).

In our studies of assessing the opportunities for symbiotic associations between rice and rhizobia, it was found that, unlike in legumes, root exudates of only a limited number of rice varieties were found to have the capability to induce *nod* gene expression, that too to a very low levels, in a restricted number of rhizobial strains. Likewise, only a few rhizobial strains were capable of inducing root hair deformation, to a marginal extent, in a few rice varieties. Also nodule-like structures/hypertrophies have been induced in rice, again at a very low frequencies (in 0.1 - 0.2% of the plants), by applying rhizobia either to normal roots (Rolfe, Bender 1990; de Bruijn et al. 1995), or to enzyme-treated roots in the presence of polyethylene glycol and calcium chloride (Al-Mallah et al. 1989). These nodule-like structures/hypertrophies appeared to originate from lateral root primordia (Cocking et al. 1994; de Bruijn et al. 1995).

The limited ability of rice root exudates to induce *nod* gene expression in rhizobia or rhizobia to induce the production of nodule-like stuctures on rice plants may partly be due to either reduced rhizobial expression of Nod factors in the presence of rice root exudates, or where Nod factors are produced then they are unable to effectively promote the nodulation processes (Ladha, Reddy 1995). Therefore, initially it is important to determine whether rhizobia are unresponsive to rice exudates due to the absence of appropriate flavonoids which trigger *nod* gene expression or to the presence of some substances in the root exudate that are inhibitory to *nod* gene expression. Such information is essential for the genetic manipulation of rice for enabling it to release of appropriate flavonoids in the root exudate. If the rhizobia are responsive to root exudate, then it becomes imperative to test the responsiveness of the rice root to Nod factors (Ladha, Reddy 1995). Any root hair curling/induction of cortical cell division implies that rice has Nod factor receptors to initiate nodule formation in response to rhizobia. So far it is not known whether Nod factors are recognized by rice although there are reports indicating that Nod factors are able to elicit mitosis and developmental responses in non-legume dicots like carrot and tobacco (de Jong et al. 1993; Yang et al. 1994). If the Nod factors do not elicit any responses in rice roots, then it is essential to ascertain whether it is due to the absence of receptors for the recognition of Nod factors or because of the degradation of the Nod factors by root chitinases.

In legume-*Rhizobium* symbiosis, the host plant provides the genetic information for the development of specialized structures such as infection threads as well as nodules. The role of the rhizobia is to trigger the host plant genes to facilitate infection and help create a niche for N_2 fixation (Denarie, Cullimore 1993). Studies of interactions between rhizobia and rice roots so far have not revealed any formation of infection threads through root hairs or the development of intricate network of infection threads in the root cortex. It is possible that cereals such as rice do not possess the genetic traits to support the formation of sophisticated infection threads and genuine nodules on these plants (J. Denarie, personal communication), but at the same time there is little reason to suppose that rice completely lacks genetic background to support the formation of nodule-like structures. Development of lateral root-derived nodular structures, albeit to a lesser extent, in certain rice varieties in response to rhizobial inoculation is a piece of evidence on this point. Besides, for rhizobial infection in rice, development of a sophisticated infection-system favouring bacterial entry through root hair may not be necessary. A process of *Rhizobium* infection through primitive "crack-entry" mode may, in fact, facilitate bypassing the cellular machinery needed for sophisticated type of infection through root hair.

An intercellular infection of nodule-like structures by rhizobia was observed in rice (Rolfe, Bender 1990). It is not known whether Nod factors elicit responses in rice and needed for intercellular infection, although in legumes the rhizobial production of Nod factors is required for all types of infection including "crack-entry" (J. Denarie, personal communication). However, if the root does not respond to Nod factors, then it becomes necessary to assess whether regulatory nodulin genes or some other specific genes would have to be transferred to rice.

It would also be important to understand more about factors that trigger defense responses in rice and to ensure that this response is not elicited by a putative microsymbiont (Bennett, Ladha 1992). A regulated supply of oxygen to rhizobia is essential for maintaining nitrogenase activity in legumes nodules, hence a leghaemoglobin-like system may be necessary also in rice to support N_2 fixation by rhizobia. Actually, one reason for optimism is that globin genes are widespread in non-leguminous plants, and encoding plant haemoglobins of cryptic function (Landsmann et al. 1986). However, it is essential to ascertain whether these globin genes are expressed at the site of symbiosis (see Jacobsen-Lyon, 1995) and the products of these genes function as oxygen regulators. If not, necessary amendments have to be made to incorporate a leghaemoglobin-like system to support symbiotic N_2 fixation in rice.

DEVELOPMENT OF SYMBIOSIS VIA COCULTURE

The problem of inducing symbiosis in rice could be addressed through regeneration of rice plants from cell lines possessing amenable properties to coexist with the diazotroph. This could be achieved by coculturing rice cells (in tissue culture) with a suitable diazotroph to encourage the production and proliferation of populations of plant cells having ability to grow in association with the selected diazotroph. The resultant populations of rice cell lines possess novel properties enabling them to coexist with the diazotroph. If such cells are made to regenerate into plants, then in all probability the regenerated plants may possess the capabilities to form symbiotic associations with the diazotroph without exhibiting host-specific barrier. Indeed, such an approach was successfully employed in producing symbiotic associations between cyanobacteria and alfalfa (Korzhenevskaya et al. 1985) and tobacco (Gusev et al. 1986) plants. However, it is essential to assess the stability of the 'acquired' symbiotic trait of the plants derived through such a process. For effective transmission and stable maintainance of diazotroph in symbiotic association it will be desirable if the symbiotic diazotroph is associated with the plant throughout its life cycle.

SYMBIOTIC NITROGEN FIXATION AFFECTS YIELD ?

Nitrogen-fixing symbioses in rice may be such a strong sink for photosynthate that yield would be affected. If so then genetic manipulation of rice may be needed for proper partitioning of substrates for N_2 fixation and other metabolic processes. However rice is low in protein, therefore a much lower rates of N_2 fixation than in legumes will be required, with less drain on the plant's photosynthates. Another important factor to consider is that the actual rice yields are much lower than their maximum genetic potential, therefore the BNF trait may have little affect at present yield levels.

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ADVANCES IN AGRONOMY AND ECOLOGY OF THE *AZOSPIRILLUM* / PLANT ASSOCIATION

OKON, Y., ITZIGSOHN, R., BURDMAN, S. AND HAMPEL, M.
Department of Plant Pathology and Microbiology and The Otto Warburg Center for Agricultural Biotechnology. Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot, 76100, ISRAEL.

Introduction

Various soil bacteria that are capable of exerting beneficial effects on plants in culture or in a protected environment have potential for use in agriculture and can lead to increased yields of a wide variety of crops (Okon and Hadar, 1987), are known as Plant Growth Promoting Rhizobacteria (PGPR) (Kloepper, 1994). It has been well established that the nonspecific associative N_2-fixing rhizobacteria of the genus *Azospirillum* may favor the development and crop yield of different host plants under appropriate growth condition (Okon and Labandera-Gonzalez, 1994).

Taxonomy of the genus *Azospirillum*.

Based on rRNA:DNA hybridizations and cataloging of the 16s rRNA, *Azospirillum* was shown to belong to group 1 of the alpha-subclass of the *Proteobacteria* in the rRNA superfamily IV. This branch is part of an rRNA cluster consisting of three other rRNA branches including species of the genus *Aquaspirillum* and *Rhodospirillum* (Gillis and Reinhold-Hurek, 1994).

The physiological and genetic properties of the five described *Azospirillum* species *(A.brasilense, A. lipoferum, A. amazonense, A. halopraeferens* and *A. irakense)* were recently reviewed in detail (Gillis and Reinhold-Hurek, 1994, Hartmann and Zimmer, 1994, Michiels *et al*, 1994). Some of the current trends in research of *Azospirillum* physiology are briefly described.

Polyhydroxyalkanoate analysis in *Azospirillum*

A wide variety of different types of microorganisms are known to produce intracellular energy and carbon storage compounds, generally described in the past as being poly–β–hydroxybutyrate (PHB). In recent years it has been found that in most cases the polymers are polyhydroxyalkanoates (PHA's) comprising copolymers with different alkyl groups at the β–position (Brandl *et al.*, 1990). Understanding the role that PHA's play as an internal storage polymer on survival, proliferation, N_2 fixation and plant growth promotion in the rhizosphere, is of fundamental importance in microbial ecology (Okon and Itzigsohn 1992).

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Azospirillum was grown on a variety of carbon sources in order to determine its production potential for different PHA's. Analyses using GCMS revealed that none of the samples contained any detectable β–hydroxyvalerate. *Azospirillum* did not grow on any n-alkanoic acid with a carbon chain above C-3 i.e. from butyrate to decanoate, or any β-hydroxyalkanoate with a chain length above that of β–hydroxybutyrate. When compared with other species, *A. brasilense* produced between 1.5 and 3 fold PHB compared to *A. lipoferum* (Itzigsohn *et al.*, 1995).

In contrast to other bacterial species such as *Alcaligenes eutrophus* and *Pseudomonas oleovorans*, *A. brasilense* does not produce copolymers of hydroxyalkanoates when grown under different sources and C:N ratios. Rather, only homopolymers of PHB are detected comprising up to 70% of the cell dry weight (Itzigsohn *et al.*, 1995). To date, mutants lacking PHA synthase activity in *A. brasilense* have not been available .

Thus isolation of the *A. brasilense phaC* gene or gene cluster could be instrumental as a first step towards the production of PHA$^-$ mutants. These PHA$^-$ mutants could enable an *in situ* assessment of PHA significance in rhizosphere colonization. When probed with a hexamer-labelled *Alcaligenes eutrophus phaC* gene, a distinct hybridization pattern to total *A. brasilense* DNA was obtained establishing the presence of a similar sequence (R. Itzigsohn- unpublished). We are currently carrying out experiments to obtain *A. brasilense phaC*$^-$ mutants.

Motility and chemotaxis

A. brasilense shows both chemotaxis and chemokinesis in response to temporal gradients of some chemoeffectors (organic acids, sugars and amino acids) by an alternative chemosensory pathway based on the metabolism of the chemoeffectors. No methylation-dependent pathway for chemotaxis could be detected in *A. brasilense* (Zhulin and Armitage,1993).

In the rhizosphere, it may be advantageous to respond positively to any compounds that increase metabolic rates, such as organic acids and oxygen, rather than relying on chemicals sensed via the dedicated methyl-accepting chemotaxis proteins (MCPs). Low oxygen concentrations typical of the rhizosphere are seen to be one of the main stimuli that attract bacteria to plant roots. Aerotaxis in the rhizosphere would be expected to provide a real advantage for bacteria in search of a source of nutrients and in competition with other microorganisms (Okon 1985, Zhulin and Armitage, 1993).

Non-motile *A. brasilense* mutants were recently obtained lacking the polar flagellum that is primarily used for swimming (Croes *et al.*, 1993). Studies carried out with this mutant, purified polar flagella, and in the presence of antiserum against the polar flagellum filament protein (Fla 1), showed evidence that adsorption of *A. brasilense* to wheat roots is mediated by its polar flagellum. Moreover, purified polar flagella adsorbed to wheat roots. (Croes *et al.*, 1993).

Mutants impaired in motility and chemotaxis have been shown to exhibit a strongly reduced wheat root colonization ability (Vanderleyden *et al.*, 1994).

Production of plant growth promoting substances

Phytohormone synthesis by *Azospirillum* is proposed to influence the host root respiration rate and metabolism and root proliferation, with a concomitant mineral and water uptake in inoculated plants (Fallik *et al* 1994). It has been demonstrated that *Azospirillum* is capable of producing indole-3-acetic acid (IAA) via multiple IAA biosynthetic pathways: amino transferase, indole-3-acetamide and by a tryptophan-independent pathway(s) (Prinsen *et al.*, 1993, Bar and Okon, 1993; 1995, Sanchez-Alonso *et al.*, 1992, Costacurta *et al.*, 1994). The production of gibberellin GA, GA_3 and iso-GA_3 in cultures of *Azospirillum lipoferum* was demonstrated by GC-MS with selected ion monitoring (SIM) (Bottini *et al.*, 1989).

In corn seedlings inoculated with *Azospirillum* ,relatively higher amounts of free (compared to conjugated forms) active IAA, indole butyric acid (Fallik *et al.*, 1994; Fulchieri *et al.*, 1994) were detected when compared to non-inoculated controls. In the non inoculated roots seedlings GA_3 was found after hydrolysis of a fraction expected to contain glucosyl conjugates (with less phytohormone activity)(Fulchieri *et al.*, 1994).

It appears that the presence of *Azospirillum* in the rhizosphere affects the metabolism of endogenous phytohormones in the plant. It is not known, however, if the phenomenon is due to free phytohormone production by the bacterium or by the elicitation or activation of conjugate phytohormone hydrolysis by the root tissue .

By screening Tn5 mutants, some excreting either high or very low amounts of IAA, those overproducing amino acid aminotransferases were obtained. From studies carried out with the above mutants, it was concluded that *Azospirillum* possesses more than one copy of the genes involved in IAA biosynthetic pathways (Castelum-Reynoso *et al.*, 1994). No mutants completely lacking production of IAA have so far been isolated, however, when comparing low IAA with high IAA producing strains a reduced ability in wheat root system promotion (root hairs and lateral roots) was observed (Barbieri and Galli 1993).

Modern methodologies for studying *Azospirillum* ecology

Taxonomic and ecological studies of *Azospirillum* (identification in the soil and rhizosphere) can be performed using 16s and 23s-rRNA sequence homology, restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) technique, amongst others. The above methods are expected to give new insight into *Azospirillum* colonization and activities in the rhizosphere (Fancelli *et al.*, 1995, Hartmann *et al.*, 1994).

Molecular tools for *in situ* detection of *A. brasilense* have recently been developed including monoclonal antibodies against *A. brasilense* Sp7 and Sp 245 (Schloter *et al.*, 1993) as well as phylogenetic oligonucleotide probes (Hartmann *et* *al*., 1994). The above techniques have been applied to demonstrate a different colonization pattern of inoculated wheat roots in axenic agar tubes and in field lysimeters, using chemiluminescence ELISA and immunogold labelling. While Sp 245 entered the interior and invaded the central vascular system 2 weeks after inoculation, *A. brasilense* Sp7 remained on the root surface (Hartmann *et al*., 1994). The monoclonal antibodies were also used for a direct reisolation of *A. brasilense* Sp7 with the magnetic column separation (MACS) technique. A strain-specific identification was possible by restriction pattern analysis and pulsed field electrophoresis (Hartmann *et al*., 1994).

Oligonucleotide probes were developed for all 5 species of *Azospirillum*. They derived from the hypervariable region of 23s rRNA. The oligonucleotide probes for *A. brasilense* were used for whole cell labelling with fluorescence labelled probes. An *in situ* identification of bacteria in the rhizosphere with fluorescence labelled cells was possible with a confocal scanning laser microscope (Hartmann *et al*., 1994, Schloter *et al*., 1993).

By using GUS and lacZ reporter systems (*nif*HA-*gus*A fusion) it was found in gnotobiotic systems that *A. brasilense* colonizes the root hair zone and the sites of emerging root laterals. (Vande-Broek *et al*., 1993, Arsene and Elmerich 1994), and that nitrogen fixation and its regulation occurs in inoculated wheat roots, mainly under low oxygen partial pressures and by the addition of external energy sources (Vanderleyden *et al*., 1994).These studies confirm previous observations carried out by classical microbiological methods (Okon, 1985).

Conclusions from Field Experiments

By evaluating worldwide data accumulated over the past 20 years on field inoculation experiments with *Azospirillum*, it can be concluded that these bacteria are capable of promoting the yield of agriculturally important crops in different soils and climatic regions. Various strains of *A. brasilense* and *A .lipoferum* have been used to inoculate cultivars of different species of plants. However, it is difficult to accurately estimate the percentage of success due to *Azospirillum* inoculation. The data indicates 60-70% occurrence of success with statistically significant increases in yield in the order of 5-30%. For details and references see Okon and Labandera-Gonzales (1994).

Interaction of *Azospirillum* with the *Rhizobium*-legume symbiosis

The effects of co-inoculation with both *Rhizobium* and *Azospirillum* on the leguminosae have received increasing attention over recent years. Positive effects of combined inoculation have been reported for different legumes (Itzigsohn *et al*., 1993; Volpin and Kapulnik, 1994). The beneficial response of dually inoculated plants was related to favorable influence of *Azospirillum* on nodule number, development, dry weight, and N_2 fixation.

The effect that *Azospirillum* has on nodulation and on the specific activity of nodule N_2 fixation leading to growth promotion may be attributed to early nodulation, an increase in nodule total number and/or a general improvement in mineral and water uptake by the roots .

Unlike gnotobiotic systems, the beneficial response achieved in field and greenhouse studies were in most cases obtained following the simultaneous application of *Azospirillum* and *Rhizobium* (Itzigsohn *et al.*, 1993; Volpin and Kapulnik, 1994). Furthermore, the promoted legume growth following *Azospirillum* inoculation was preceded by enhanced nodulation of the plants.

Our group is presently studying the effects of *Azospirillum* application in the *Phaseolus vulgaris -Rhizobium* system. In this particular symbiosis, the nodulation process is generally slow and the plant has a short growth cycle. Thus, N_2 fixation does not contribute significantly to the N nutrition of the plant. In the first experiments that were carried out, it was observed that *Azospirillum* at 10^8 cfu.ml^{-1} stimulates nodule formation and N_2 fixation in *Phaseolus vulgaris*. The number of upper nodules (those that formed earlier) is generally significantly higher in plants co-inoculated with *Rhizobium* and *Azospirillum* at 10^8 cfu.ml^{-1} in comparison with the control plants (inoculated with *Rhizobium* alone).

We are currently investigating the effects of *Azospirillum* on the production of plant signals that activate nodulation genes in *Rhizobium*.

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N_2 FIXATION IN SUGAR CANE: THE ROLE OF *ACETOBACTER DIAZOTROPHICUS*

BODDEY, R.M., REIS, V.M. URQUIAGA, S., DA SILVA, L.G.,
REIS, F.B. BALDANI, J.I. AND DOBEREINER, J.
Centro Nacional de Pesquisa de Agrobiolgia (EMBRAPA-CNPAB), Km47,
Seropedica Itguai 23451-970, Rio de Janerio, Brazil

Introduction

Almost 4 million cars in Brazil are fueled by hydrated (95%) ethanol produced from sugar cane and the remaining 6 to 8 million light vehicles run on gasohol (gasoline mixed with 15 to 22% ethanol). The bio-ethanol programme has great environmental advantages: Not only does the addition of ethanol to gasoline obviate the need for tetra-ethyl lead, but engines running on hydrated alcohol produce less carbon monoxide, nitrogen oxides and hydrocarbon in the exhaust gases than conventional gasoline engines (Boddey, 1993). Furthermore, unlike gasoline or other fossil fuels, the combustion of this biofuel makes no net contribution to global CO_2 emissions as the carbon was fixed from the atmosphere by the sugar cane crop. However, when considering the CO_2 emission budget, the amount of fossil fuel used to grow the crop must be taken into account. This includes diesel fuel for agricultural machinery and transport of cane to the mill, as well as the energy used to process the cane and distill the ethanol. As sugar cane in Brazil is still largely harvested manually, and the energy to power the mills and distilleries is all provided from burning bagasse, this ratio of energy value of the ethanol to fossil fuel used in its production (the energy balance) is very favourable (approximately 4.5).

Fertilizer inputs can make a very significant impact on the energy balance. N fertilizer requires 6 - 7 times as much energy (principally natural gas) to produce as P or K fertilizers (Da Silva *et al.*, 1978), and the tradition in Brazil of using low N fertilizer inputs (mean 60 kg N ha^{-1} yr^{-1}) on sugar cane also helps to maintain a favourable energy balance. For example if an additional 100 kg N ha^{-1} yr^{-1} of N fertilizer were applied (a conservative addition for cane producers in other countries) the net energy balance would be lowered to 3.3, and if N fertilizer were to be eliminated entirely the balance would increase to close to 6.0. (Boddey, 1995).

In Brazil the amount of N applied as fertilizer is generally less than that removed from the field when the crop is harvested. Despite this, cane yields do not decline even on soils that have been continuously cropped with sugar cane for many decades, even centuries. These observations, amongst others, suggested that there might be inputs of biological nitrogen fixation (BNF) associated with this crop.

BNF contributions

Significant nitrogenase activity associated with the roots of sugar cane was detected using the acetylene reduction assay by several authors (Döbereiner *et al.*, 1972;

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Purchase,1980) but the first attempt to quantify the actual contribution of BNF to whole plants was made by Ruschel *et al.* (1975) who reported significant incorporation of $^{15}N_2$ gas into intact 90 day-old sugar cane plants. However, only when N balance and ^{15}N dilution techniques were used could the contribution of BNF be evaluated over the whole plant growth cycle. The ^{15}N-aided N balance study performed in large pots (64 kg soil pot^{-1}) of Lima *et al.* (1987) showed that more than 60 % of the N accumulated by the Brazilian commercial cane variety CB 47-89 was derived from N_2 fixation -equivalent to over 150 kg N ha^{-1} yr^{-1} on the basis of 15000 plants ha^{-1}. These large contributions were confirmed, again using the N balance and ^{15}N dilution techniques, in an experiment where 10 sugar cane varieties were grown in a large concrete tank (20 x 6 m) filled with ^{15}N-labelled soil (Urquiaga *et al.*, 1992). In this case the commercial varieties CB 45-3 and SP 70-1143 and the *Saccharum spontaneum* variety, Krakatau, BNF contributed the equivalent of 170 kg N ha^{-1} yr^{-1} or more over a 3 year period.

It should be pointed out that P, K and micronutrient fertilization in this experiment were optimum, and the experiment was irrigated throughout the year. Year-round irrigation, which is rare in Brazil, seems to be a key to high BNF inputs. On one sugar cane plantation in the north-east of Rio de Janeiro State, it was found that the application of 92 kg N ha^{-1} yr^{-1} as urea had no effect on cane yields and production averaged 97 t ha^{-1} with no N fertilizer (Boddey, 1995). Where irrigation is not available, reponses to N fertilizer are frequently reported, but in a long-term (9 year) N balance study on a plantation in Pernambuco (NE Brazil) the data suggested that a mimimum of between 32 and 54 kg of plant N were derived from BNF in the variety CB 45-3, even though N losses from the system were not quantified (Oliveira *et al.*, 1994).

To date all studies to quantify BNF contributions to sugar cane have been performed on Brazilian varieties. All the commonly used varieties in this country were bred under very low N fertilizer regimes which may account for the high contributions and why it is usually found necessary in other countries to add N fertilizer at rates varying from 150 to 300 kg N ha^{-1} yr^{-1} to attain maximum yields.

N_2-fixing bacteria associated with sugar cane

Even before good data were available on the BNF contributions of sugar cane many species of N_2-fixing bacteria including *Beijerinckia*, *Azospirillum*, *Azotobacter*, *Erwinia*, *Derxia*, and *Enterobacter* spp. were isolated from the rhizosphere, roots, stems and even leaves of sugar cane plants (Döbereiner, 1961; Purchase, 1980; Graciolli *et al.*, 1983). However, more recently a new species of N_2-fixing bacteria, *Acetobacter diazotrophicus*, was found to occur in high numbers in the roots and stems of sugar cane (Cavalcante, .. Döbereiner, 1988; Gillis *et al.*, 1989). This diazotroph was originally isolated in a semi-solid medium based on sugar cane juice and is a small, Gram-negative, aerobic rod showing pellicle formation (microaerobic aerotaxis) in N-free semi-solid medium with 10% sucrose. After 7 to 10 days growth it forms a thick surface pellicle. Best growth occurs with high sucrose or glucose concentrations (10%) and strong acid production results in a final pH of 3.0 or less but growth and N_2 fixation can continue at this pH for several days (Stephan *et al.*, 1991). The bacterium possesses no nitrate reductase and N_2 fixation is not affected by high levels (25mM) of NO_3^-. Also NH_4^+ causes only partial inhibition of nitrogenase especially when the bacteria are grown at high sucrose concentrations (100 g l^{-1} - Teixeira *et al.*, 1987; Boddey *et al.*, 1991). The bacteria also shows a much higher O_2 tolerance than

Azospirillum spp. (Reis *et al.*, 1990). This may be important in that the bacteria may continue to fix N_2 within the plant even if the pO_2 is not much lower than that in the air.

The bacterium has been found in many sugar cane varieties in several regions of Brazil as well as Mexico, Australia and Cuba (Fuentes-Martinez *et al.*, 1992; Li, Macrae, 1991; Dong *et al.*, 1994). In Brazil it was not found in soil between rows of sugar cane plants or roots from 12 different weed species taken from cane fields (Döbereiner *et al.*, 1988), nor in other grasses taken from sugar cane fields in Australia (Li, MacRae, 1991). However, *A. diazotrophicus* has been isolated from a few samples of washed roots and aerial parts of *Pennisetum purpureum* cv. Cameroon, and from sweet potatoes (Döbereiner *et al.*, 1988; Paula *et al.*, 1989).

More recently other significant populations of endophytic N_2-fixing bacteria have been isolated in from the tissues of sugar cane plants (Döbereiner, Baldani, Reis - unpublished data). Investigations are most advanced in the case of two species of *Herbaspirillum* (*H. seropedicae* and *H. rubrisubalbicans*) and this information was recently reviewed by Boddey (1995) but for reasons of brevity cannot be discussed here. However, there is no evidence available to suggest that these other diazotrophs are any less important in their contributions of BNF to sugar cane. Because of their preferential use of organic acids, a population of these diazotrophs associated with *A. diazotrophicus* (which produces acetic acid as a product of sugar fermentation), may explain how the pH of the intercelluar fluid of the cane stem remain stable at approximately 5.5.

Infection of sugar cane by *Acetobacter diazotrophicus*

All samples of sugar cane taken from the field and studied at our Centre have been found to contain *A. diazotrophicus*. To study the infection therefore it has been necessary to utilize monoxenic micro-propagated plantlets (James *et al.*, 1994). Such seedlings were not infected by *A. diazotrophicus* by traditional root inoculation methods when transplanted to carbon-free medium. However, it was found that when micropropagated plants were transferred from the tissue culture medium (MS medium with 20g sucrose l^{-1}) to the same medium diluted by a factor of 10, and inoculated with a suspension of 10^7 cells ml^{-1} of *A. diazotrophicus*, the plants remained healthy and all tissues became extensively colonized by the bacteria. This study was performed using both optical and electron microscopic techniques and immuno-gold labelling. On the root surface the bacteria was found especially on the root tips and around lateral root junctions and the bacteria appear to infect the plants at these sites. Within the roots *A. diazotrophicus* was observed in apparently intact, enlarged epidermal cells, and at the base of the stem within xylem vessels through which the bacteria appear to migrate upwards in the transpiration stream to the shoot tissues.

Dong *et al.* (1994) isolated *A. diazotrophicus* from the apoplastic fluid (fluid from the intercellular spaces of the stem parenchyma) of sugar cane taken from the field or pots of soil, and reported counts of 10^4 cells ml^{-1} of this bacterium, which was almost certainly an underestimate as they counted the bacteria on solid medium. They also visualized bacterial cells in the intercellular spaces of the stems using transmission and scanning electron microscopy and presented convincing evidence to suggest that these bacteria were *A. diazotrophicus*, providing confirmation of their endophytic habit.

It should be stressed that infection of plants grown in normal (carbon-free) plant nutrient solutions or in soil seems to be very rare as *A. diazotrophicus* will not survive in the medium without an abundant supply of sucrose. Paula *et al.* (1991) found that the difficulty of infection of plants grown in soil or vermiculite can be overcome by co-inoculation with VA mycorrhizal fungi, especially originating from fungal spores infected with the bacteria. This diazotroph has also been isolated from pink mealy bugs (*Saccharococcus sacchari*) which feed on the meristematic regions of sugar cane (Ashbolt, Inkerman, 1990), and this has been suggested as a further mechanism of plant to plant transfer of the bacterium (Cabellero-Mellado, Martinez-Romero, 1994).

Genetic diversity of *Acetobacter diazotrophicus*

Caballero-Mellado and Martinez-Romero (1994) examined the genetic diversity of 24 isolates of *A. diazotrophicus* from diverse geographical regions of Mexico and Brazil using multilocus enzyme electrophoresis, DNA fingerprints, and plasmid and *nif*HDK patterns. They concluded that there was an extremely low genetic diversity amongst the strains, which they attributed to the fact that this obligate endophyte is only found in the relatively constant environment within sugar cane and a few other vegetatively propagated plants. Its unusual phenotypic characteristics of significant growth only in high sugar concentrations, its lack of nitrate reductase and capacity to grow and fix N_2 at very low pH, confirm its adaption to one very specific habitat. As the bacteria is propagated from plant to plant within stem pieces (setts), or possibly by mealybugs or via VA mycorrhizal fungi, it does not require the ability to survive in the very variable conditions of nutrition, moisture and pH experienced in soil.

Understanding the diazotroph/sugar cane association

Both *A. diazotrophicus* and *Herbaspirillum* spp. have been shown by microscopy to inhabit the interior of cane plants (Olivares *et al.*, 1993; Dong *et al*, 1994; James *et al.*, 1994). Other diazotrophs including *Azospirillum amazonense* and *Burkholderia* have also been isolated from inside sugar cane plants (Döbereiner *et al.* unpublished data), but which of these bacteria make the most important contribution to BNF is not known.

In order to resolve this question, techniques are required which will specifically quantify the populations of the different diazotrophs. To this end at our Centre two different strategies have been adopted based on the use of the polymerase chain reaction (PCR) and the enzyme-linked immuno-sorbent assay (ELISA).

A species-specific gene probe was developed for *A. diazotrophicus* based on the 23S ribosomal DNA (Reis *et al.*, 1993). This probe can be used for definitive identification of isolates, and, more importantly for our objectives, as a primer for the PCR reaction. Initially, by adding, or not, viable cells of *A. diazotrophicus* to macerated samples of sugar cane roots and stems before submitting them to the PCR amplification, it was established that the presence of plant material did not interfere with the amplification. Subsequently, samples taken from small sugar cane plants (infected with *A. diazotrophicus*) derived from micro-propagated plantlets were inoculated in a rich culture medium and an aliquot of this suspension was used for the amplification. This also gave positive results and recently the technique has applied directly on untreated macerates of sugar cane plants taken from the field. While this technique can specifically identify *A. diazotrophicus*, and similarly could be applied to the identification of the two species of *Herbaspirillum*, or other diazotrophs, its use to enumerate the viable cells of each species using techniques such as that described by Sykes *et al.* (1992) may turn out to be extremely laborious.

The ELISA technique has two major prerequisites: the use of a antibody that reacts with all possible strains of the target species and a lack of cross-reaction with antigens of plant origin or related bacterial species. The primary polyclonal antibody was produced by inoculating a rabbit with cells of the *A. diazotrophicus* strain PRJ-2 pretreated at 90°C to eliminate flagellar proteins (Reis, 1994). Using this antibody for indirect ELISA, 10^7 cells ml^{-1} of 25 different *A. diazotrophicus* strains all showed absorbances at 405 nm of between 0.61 and 0.90. The same number of cells of other species of *Acetobacter* (*A. hansenii*, *A. aceti*, *A. pasteurianus* and *A. liquefaciens*) showed absorbances between 0.23 and 0.49, various other diazotrophs including species of *Herbaspirillum*, *Azospirillum*, *Klebsiella* and *Burkholderia* all showed absorbances below 0.07 and 10 unidentified bacteria isolated from sugar cane less than 0.15. These results suggested that this primary polyclonal antibody could be used with ELISA to quantify numbers of *A. diazotrophicus* in extracts of sugar cane plants with little interference from the presence of other bacteria.

The antigen of this serum was found to be a 27 kDa protein localized on the external membrane of the bacteria (Reis *et al.*, 1994), and the above results suggest that this protein was present in all 25 isolates which is consistent with the narrow genetic diversity reported above.

Conclusion

Firm evidence is now available that several Brazilian sugar cane varieties can benefit from large contributions of plant-associated BNF. The present challenge is to understand which species, or combination of species, of N_2-fixing bacteria from amongst those isolated, represent the principal agents of N_2 fixation. Initially it is necessary to specifically quantify the diazotroph populations in the different plant tissues at different stages of plant ontogeny in cane varieties with different capacities to obtain N from BNF. For this the ELISA, and perhaps PCR, techniques would appear to be those most suitable and these studies need to be performed in conjunction with techniques (^{15}N) for the quantification of BNF. Subsequently, the use of optical and electron microscopy with immunological visualization techniques may reveal colonization patterns, or the presence of symbiotic structures, responsible for the high BNF contributions observed.

These studies have a great importance, not only in the viabilization of an efficient, pollution free source of biofuel from sugar cane, but it may be possible to apply the knowledge obtained to establish efficient N_2-fixing systems in grass and cereal crops, reducing the need for N fertilizer produced from fossil fuels.

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THE BROAD HOST RANGE OF TWO BEAN NODULATING RHIZOBIA, *RHIZOBIUM TROPICI* CIAT899 AND *RHIZOBIUM* SP. BR816: FIELD STUDIES AND GENETIC ANALYSIS

VLASSAK, K. (1,2), VAN RHIJN, P. (3), FRANCO, A.A. (2),
WAELKENS, F. (1), VERRETH, C. (1), LUYTEN, E. (1) AND
VANDERLEYDEN, J. (1)
(1) F.A. Janssens Laboratory of Genetics, Catholic University of Leuven, B-3001 Heverlee, Belgium
(2) CNPAB/EMBRAPA, Itaguaí, RJ, Brazil
(3) Department of Biology, UCLA, 405 Hilgard Ave, 90024-1606 Los Angeles, USA

Introduction

Common bean (*Phaseolus vulgaris* L.) is an important crop in the tropics and subtropics, supplying about 20% of the protein intake per person (Van Rheenen, 1982). Inoculation of beans with appropriate *Rhizobium* spp. strains has produced increased yields (Rennie, Kemp, 1983; Duque et al., 1985; Asad et al., 1991). However, a poor understanding of the environmental factors affecting rhizobial ecology and of the mechanisms important in this bean-bacteria interaction often results in failure of the inoculated strains to form an effective symbiosis with this host (Moxley et al., 1986; Ramos, Boddey, 1987; Thies et al., 1991; Wolff et al., 1991). A good *Rhizobium* spp. inoculant strain is able to adapt well in soil, to compete with other rhizobia for nodule formation and to initiate an effective N_2 fixation.

Bacteria capable of eliciting nodules on *P. vulgaris* roots form a very heterogenous group: besides the narrow host range species *R. leguminosarum* bv. *phaseoli* and *R. etli* (Segovia et al., 1993), the formely called *R. leguminosarum* bv. *phaseoli* type I strains (Martínez-Romero et al., 1991), beans are also nodulated by various rhizobial species with a broad host range. Among them the stem nodulating *Sesbania* symbiont, *Azorhizobium caulinodans* (Waelkens et al., 1995), *Rhizobium* sp. NGR234 (Lewin et al., 1987), *R. tropici* (Martínez-Romero et al., 1991) and *Rhizobium* sp. BR816 (van Rhijn et al., 1993).

R. tropici, previously referred to as *R. leguminosarum* bv. *phaseoli* type II, is native to South America but has also been encountered in nodules of *P. vulgaris* from acid soils in Kenya (Martínez, 1994). Besides their ability to nodulate bean, strains of this species also nodulate a wide range of tropical trees, including *Leucaena* spp. They are genetically stable, retaining their symbiotic plasmid after prolonged incubation at 37°C. Some are heat-tolerant (Karanja, Wood, 1988) or acid- and aluminium-resistant (Graham et al., 1982; Cunningham, Munns, 1984; Vargas, Graham, 1988). *R. tropici* subgroup IIB (Martínez-Romero et al., 1991) in contrast to subgroup IIA and to *R. etli*, does grow on LB medium, is resistant to various antibiotics and to heavy metals. These abilities might give strains of this subgroup and advantage for establishing a stable symbiosis in the field, even in adverse environmental conditions such as high temperature or acid soils.

Phenotypical analysis revealed that *Rhizobium* sp. BR816 shares a lot of characteristics common to *R. tropici*. However, sequence analysis of the 16S rRNA

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gene showed a closer relationship to *R. meliloti* (Martínez, 1994).
R. tropici IIB CIAT899 and *Rhizobium* sp. BR816 have shown to be very efficient for N_2 fixation at optimal temperatures (28°C/23°C day/night) as well as at high temperature (38°C for 5h day; Strallioto et al., 1992; Hungria et al., 1993). Their ability to interact with a wide variety of tropical legumes is an additional characteristic which might provide an advantage in rhizosphere survival and competition for nodule occupancy. However, little is known about the behaviour of these broad host range strains in soil since most studies on survival and competition in bean rhizosphere have focussed on type I strains.

We used successive bean cultures to study the competition and persistence of inoculated *R. tropici* CIAT899 in the field and to measure the impact of bean rhizosphere and inoculation on the development of the indigenous *Rhizobium* spp. population. Some determinants for the broad host range of *R. tropici* CIAT899 and *Rhizobium* sp. BR816 were characterised. Various *nodD* alleles were identified in CIAT899 and in BR816. In the latter strain, the different *nodD* alleles contribute differently in *nod* gene induction and nodulation. We have shown that the *nodS* gene of CIAT899 is important for nodulation of *Leucaena leucocephala* and *P. vulgaris* while the *nodO* gene of BR816 seems to play a role in the nodulation of *L. leucocephala* and *Trifolium repens*.

Competition and persistence of *R. tropici* during successive bean cultures

During 3 successive years, beans (*P. vulgaris* cv. Carioca) were sown on a planosol at the experimental field of CNPAB/EMBRAPA in the state of Rio de Janeiro, Brazil. They were inoculated with strains of *R. tropici* (CIAT899 or F98.5), *R. etli* (W16.3SB) or a mixture of a *R. tropici* and a *R. etli* strain (Table 1). At the 2nd and 3rd planting, seeds in only half of each plot were reinoculated while the other half was sown without reinoculation. This enabled us to measure the competitivity of the inoculated *R. tropici* strains with the indigenous soil population (reinoculated plots) as well as the adaptation and survival of the inoculated *R. tropici* strains after 2 and 3 years (not reinoculated plots).

Our experimental site had not been planted with *P. vulgaris* for the past 20 years. Consequently, plant dilution estimates of the bean nodulating rhizobia initially present (Table 1) were very low (less than 30 g^{-1} dry soil). During the first culture and the subsequent fallow summer period however, the population density increased rapidly and by the second planting 10^3 bean rhizobia g^{-1} dry soil were counted. Before the 3rd planting, after 2 bean crops and 2 fallow summers, the number of bean rhizobia in the plots had reached 10^4 g^{-1} dry soil.

Table 1: Population estimates of the bean nodulating rhizobia at our experimental site before each bean culture. No significant differences were found between treatments ($p \leq 0.05$) in any year.

TREATMENTS	Bean nodulating rhizobia in log(CFU)		
	before 1st planting	before 2nd planting	before 3rd planting
Uninoculated control	<1.5	3.42	4.07
R. tropici IIB CIAT899	<1.5	2.89	4.07
R. tropici IIB F98.5	<1.5	2.94	4.36
R. etli W16.3SB	<1.5	3.17	3.45
CIAT899 x W16.3SB	<1.5	3.68	3.81
F98.5 x W16.3SB	<1.5	3.15	4.21

The percentage of nodules occupied by *R. tropici* IIB in each year is shown in table 2.

At the first planting almost no native rhizobia were present. Consequently most nodules were occupied by the inoculated strains (Table 2a). In the 2nd year (Table 2b), uninoculated plots were also nodulated. Determination of this nodule occupancy showed mostly strains of *R. etli*, *R. leguminosarum* bv. *phaseoli* and *R. tropici* IIA and less than 30% of the isolates were able to grow on LB medium.

Table 2a: Percentage of nodules occupied by *R. tropici* IIB at the first bean culture. Treatments with the same letter are not significantly different ($p \leq 0.05$). Not enough nodules were recovered from the uninoculated control plants to give a reliable occupancy determination.

TREATMENTS	Nodule occupancy by *R. tropici* IIB (in%)				
	CIAT899	F98.5	native	total	
Uninoculated control	–	–	–	–	
R. tropici IIB CIAT899	93.25	0.00	6.75	100.00	A
R. tropici IIB F98.5	4.55	95.45	0.00	100.00	A
R. etli W16.3SB	3.25	0.00	0.00	3.25	C
CIAT899 x W16.3SB	79.58	0.00	0.60	80.18	AB
F98.5 x W16.3SB	3.35	49.48	1.78	54.61	B

Table 2b: Percentage of nodules occupied by *R. tropici* IIB at the second bean culture. A significant interaction was observed between the bacterial treatments and the reinoculation. Therefore data are given separately for plots inoculated in the 1st year only and the plots reinoculated in the 2nd year. Treatments with the same letter are not significantly different ($p \leq 0.05$).

TREATMENTS	Nodule occupancy by *R. tropici* IIB (in %)				
	CIAT899	F98.5	native	total	
Inoculated at first planting only	1.56	1.71	29.00	32.27	C
Uninoculated control					
R. tropici IIB CIAT899	66.74	0.00	3.17	69.91	A
R. tropici IIB F98.5	1.56	34.35	16.44	52.35	B
R. etli W16.3SB	4.27	1.08	21.20	26.55	C
CIAT888 x W16.3SB	2.26	0.00	16.04	18.30	C
F98.5 x W16.3SB	1.22	3.68	28.53	33.43	C
Reinoculated at second planting	3.63	0.00	18.45	22.08	E
Uninoculated control					
R. tropici IIB CIAT899	89.38	0.00	0.56	89.94	A
R. tropici IIB F98.5	0.00	59.08	9.11	68.19	B
R. etli W16.3SB	8.83	0.00	16.97	25.80	DE
CIAT899 x W16.3SB	42.74	0.00	6.42	49.16	C
F98.5 x W16.3SB	0.00	29.20	10.40	39.60	CD

In a soil with almost no native bean rhizobia, *R. tropici* CIAT899 proved to be the most competitive: mixed inoculation of CIAT899 with W16.3SB in equal amounts resulted in 80% nodule occupancy by CIAT899 while inoculation of F98.5 together with W16.3SB gave 50% nodule occupancy by each strain (Table 2a). Also in the presence of a population of 10^3 g^{-1} dry soil, inoculation of CIAT899 resulted in nodule occupancy mostly by this strain (Table 2b, reinoculated). At the third planting however, with a indigenous population of more than 10^4 g^{-1} dry soil, CIAT899 was no longer competitive, resulting in a very low nodule occupancy by this strain (Table 2c).

Data from the plots which were not reinoculated at the 2nd and 3rd planting give some idea about the adaptation , survival and competitiveness of the first year inoculated strains after the fallow summer period. The *R. tropici* IIB strains used could continue to some extent for 1 year after introduction (Table 2b), but not for 2 years (Table 2c).

Table 2c: Percentage of nodules occupied by *R. tropici* IIB at the 3rd bean culture. No interaction was observed between the bacterial treatments and the reinoculation. Therefore data from plots inoculated in 1st year only and from plots reinoculated in 2nd and 3rd year were pooled. Treatmenst with the same letter are not significantly different ($p \leq 0.05$).

TREATMENTS	Nodule occupancy by *R. tropici* IIB (in %)				
	CIAT899	F98.5	native	total	
Uninoculated control	0.00	0.00	0.75	0.75	A
R. tropici IIB CIAT899	2.60	0.00	5.00	7.60	A
R. tropici IIB F98.5	0.40	10.90	12.10	23.40	A
R. etli W16.3SB	0.25	0.00	1.50	1.75	A
CIAT888 x W16.3SB	8.10	0.00	13.10	21.20	A
F98.5 x W16.3SB	1.80	7.40	9.90	19.10	A

In the presence of the host and during the following summer periods, a population of mostly *R. etli*, *R. leguminosarum* bv. *phaseoli* and/or *R. tropici* IIA increased to 10^3 in the 2nd year and to 10^4 rhizobia g^{-1} dry soil in the 3rd year, even in never inoculated plots or in plots inoculated only with *R. tropici* IIB. To test whether this indigenous developed population indeed was more competitive than CIAT899 or whether CIAT899 was saprophytically not competent and failed to adapt to environmental factors, some of these bean nodulating rhizobia were isolated from plots with a different culture and inoculation history and evaluated for their competitiveness against CIAT899 under monoxenic conditions (Table 3).

Table 3: Competition index (CI = % nodule occupancy / % in inoculum) of isolates analysed by their origin (culture history and inoculation history of the plots). CI with the same letter are not significantly different ($p \leq 0.05$).

ORIGIN		CI	
Culture	Inoculation		
bean	CIAT899	1.2715	A
bean	W16.3SB	1.0522	B
bean	without	0.8127	C
without	without	0.7411	C
culture collection		0.6432	C

The indigenous rhizobial population which had developed after 3 bean cultures in plots which were repeatedly inoculated with *R. tropici* CIAT899 was the most competitive. Also the isolates from plots with *R. etli* W16.3SB inoculation were significantly more competitive than from plots which had never been inoculated. Although almost no inoculated CIAT899 was recovered, inoculation with this strain seemed to have played a role in determining the population which developed.

Characterization of determinants responsible for the broad host range of *R. tropici* CIAT899 and *Rhizobium* sp. BR816

A possible mechanism among others, responsible for a broad host range nodulation of a certain strain is the NodD protein and its spectrum of flavonoid specificity.
We have studied the *nodD* regulatory genes for both bean symbionts (Table 4). In *R. tropici* CIAT899 five *nodD* alleles were identified of which *$nodD_1$* contributes most to the NodD activity (van Rhijn et al., 1994a). *Rhizobium* sp. BR816 contains 4 *nodD* genes which differ in their interaction with flavonoids and in nodulation. $NodD_1$ and $NodD_2$ need a coinducer for gene activation, while $NodD_3$ shows hardly any inducing activity and $NodD_4$ contains a high basal activitiy and no response to any of the flavonoids tested (van Rhijn et al., 1994b). The *$nodD_2$* and *$nodD_4$* genes of BR816 seem important for *Leucaena* nodulation while *$nodD_3$* and *$nodD_4$* appear to be responsible for nodulation of *Phaseolus* (van Rhijn et al., 1994).

Table 4: Activity of the different NodD proteins of *R. tropici* CIAT899 and *Rhizobium* sp. BR816.

STRAIN	NodD	Induction of *nodAB-uidA*[a]	Nodulation[b]	
			bean	*Leucaena*
CIAT899	$NodD_1$	flavonoids	+	+
	$NodD_2$	–	–	–
	$NodD_3$	(flavonoids)	±	–
	$NodD_4$	–	–	–
	$NodD_5$	–	±	–
BR816	$NodD_1$	(flavonoids)	±	–
	$NodD_2$	flavonoids	±	+
	$NodD_3$	–	+	±
	$NodD_4$	constitutive	+	+

[a] –: no induction by the tested flavonoids.

(flavonoids): some induction by flavonoids tested, but very little compared to other NodD proteins.

[b] +: fully developed nodules.

± 25-75% of the testplants nodulated.

–: no nodules.

The host specific nodulation genes are the most important determinants for the host range of a certain strain. The *nodSU* genes of *R. tropici* CIAT899 were extensively studied (Table 5). While *nodS* is essential for nodulation of *P. vulgaris* and *L. leucocephala*, *nodU* mutation caused a decrease in nodulation of *Leucaena* but resorted no effect on *Phaseolus*. Also the host range extension to *Leucaena*, observed with *R. etli* CE3 carrying the *nodS* genes of CIAT899 demonstrated that *nodS* is a host range determinant for nodulation of *Leucaena* (Waelkens *et al.*, 1995). Genetic analysis of *Rhizobium* sp. BR816 reveals some interesting characteristics: besides the presence of *nodSU* and possibly 3 copies of *nodEF* which code for Nod factor decorations, also a *nodO* homologous gene was identified.

Table 5: Nodulation phenotype (nodule number) of *nodS* and *nodU* mutants of *R. tropici* CIAT899.

STRAIN	Bean	*Leucaena*
CIAT899	50	5
CIAT899*nodS*⁻	0	0
CIAT899*nodU*⁻	61	2

The presence of *nodO* has sofar been reported only for *R. leguminosarum* bv. *viciae*. This gene codes for a secreted Ca^{2+} binding protein that shows homology with haemolysin (de Maagd et al., 1989). The function of NodO in the *Rhizobium*-plant interaction is unclear. Downie and Surin (1990) postulated that NodO is a host determinant for nodulation of vetch (*Vicia*) and they were able to complement a *nodE* mutation in *R. leguminsarum* bv. *viciae* with *nodO*. Also a host range extension of a *R. leguminosarum* bv.*trifolii nodE* mutant to vetch was observed.
We were able to extend the host range of the narrow host range strains *R. etli* CE3, *R. leguminosarum* bv. *trifolii* ANU843 and *R. meliloti* 1021 to *Leucaena* by introducing *nodO* of BR816. Complementation of a *nodSU* mutation in NGR234 and a *nodE* mutant of ANU843 by *nodO* was also possible. Overproduction of NodO in BR816 could extend its host range to *T. repens*. We conclude that NodO of BR816 is another determinant for nodulation of *L. leucocephala* and *T. repens*.

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INOCULANT FORMULATIONS AND APPLICATIONS TO MEET CHANGING NEEDS

SMITH R.S.
LiphaTech, Inc., Nitragin Brand Inoculants, Milwaukee, WI 53209 USA

Legume inoculants provide a means for transporting superior nitrogen-fixing rhizobia from the fermentor to the legume field. A high quality formulation and application must maintain and deliver, in a convenient and economical fashion, a high population of effective rhizobia. Soybeans are a major worldwide legume and constitute a high percentage of total inoculum use. Soybeans in the USA under intense cropping systems are being planted and managed in a changing manner to improve soil conservation. Conservation tillage is a system of cultivation and crop residue management that maintains at least 30% crop residue on the soil surface (Dickey et al., 1994). No-till, ridge-till, strip-till, and mulch-till are examples of conservation tillage. Forty-six percent of the US soybeans were under conservation tillage in 1994 with the majority of this production utilizing narrow-row planting (18-30 cm) instead of the more common 76-91 cm row width with conventional tillage (Johnson, 1994). Drills are frequently used for narrow-row plantings and, in addition, air or vacuum planters are replacing the plate planters in wide rows.

The traditional peat powder inoculant (Strijdom, Deschodt 1976; Smith, 1987) for seed application frequently does not satisfy the planting needs with these new generation planters. Peat powder is easily removed with the seed air delivery systems, and peat also interferes with the seed monitor mechanism. The addition of stickers and slurry application will improve the seed adhesion, but requires additional time and labor to the inoculation process that is already labor intensive.

Convenience and application efficiency are very important features to new inoculant formulations that are being developed and implemented to provide quality, convenience, and planter compatibility. A liquid seed-applied soybean inoculant *(Cell-Tech®* S, LiphaTech, Inc.) has been developed, which provides a quality and convenient product (Smith, 1994). The sterile liquid inoculant is packaged in a plastic bag and may be applied in either a batch treatment, or continuous application system with the aid of an applicator kit. A small, premeasured package contains 75 ml in each of two cells in a bag. Each corner of the bag is torn open at the notch before applying the inoculant to 27.2 kg of seed. A uniform moist coating of the seed is achieved before the inoculant quickly dries. A large unit contains three liters, which is sufficient to inoculate 1089 kg of soybean seeds. The bag can be removed from the shipping box, an applicator unit attached, and the bag hung by a grommet allowing the inoculant to be dispensed into a drill-fill auger which moves bulk seed into the planter or drill. The auger provides excellent mixing of the liquid

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with the seed. Inoculated seed may be planted with conventional planters and the newer air or vacuum planters without losing any inoculum from the seed. Once dried on the seed this inoculum does not interfere with the window of the planter-mounted seed monitors.

The liquid inoculant stored at 20 to 25°C maintains a population ranging from 2 x 10^9 to 5 x 10^9 cfu/ml during a 14-month period (Fig.1). When applied to the seeds and allowed to dry the recovered populations range between 433,000 to 880,000 cfu/seed (Table 1). The liquid-inoculated seed produced 11.5 nodules per plant 42 days after planting in Plainfield loamy sand low in available nitrogen and free of *Bradyrhizobium japonicum*. This is significantly better than the seed-applied peat (5.0 nodules per plant). The liquid seed inoculant yielded 2149 kg/ha, which is significantly better than the control (1575 kg/ha), and 320 kg/ha greater than the peat powder (1829) (Fig.2).

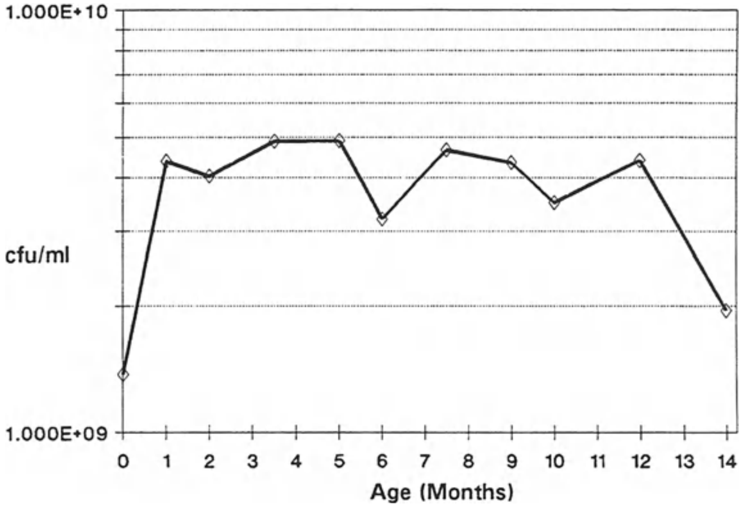

Figure 1. *Bradyrhizobium japonicum* survival in liquid inoculum (*Cell-Tech* S) stored at 20 to 25°C.

A liquid in-furrow inoculant (LIFTÔ, LiphaTech, Inc.) is also a sterile inoculant that provides convenience and quality. The concentrate (2 x 10^9 cfu/ml guarantee) is applied at a rate of 9.5 ml/100 m of row, is diluted with water, and dispensed in a volume ranging from 28 to 94 L/ha at a 76 cm row width. Liquid delivery equipment, either tractor or planter mounted, can be modified to place the inoculant in the furrow on top of the seed before the furrow is closed. The higher rate of inoculum applied in-furrow with LIFT provides fifteen times the rhizobia when compared to the liquid on seed inoculum (*Cell-Tech*). The higher dose of applied rhizobia provided better nodulation (LIFT - 19.6 nodules per plant) than the seed-applied liquid (*Cell-Tech* - 11.5 nodules per plant). In a trial in sandy soil, low in available nitrogen, and free of *B. japonicum*, the liquid in-furrow treatment also provided a greater yield (Fig. 2).

Table 1. *Bradyrhizobium japonicum* survival on seeds when applied as liquid inoculant (*Cell-Tech* S).

Sample No.	*Cell-Tech* (cfu/ml)	*B. japonicum* per seed	Time on Seed (hours)
1	6.8×10^9	880,000	1
2	4.9×10^9	510,000	1
3	4.9×10^9	771,000	1
4	5.0×10^9	833,000	1
5	4.0×10^9	489,000	1
6	4.7×10^9	433,000	48

A non-sterile peat granule (*Soil Implant*®, LiphaTech, Inc.) with a 1×10^8 cfu/g guarantee, applied at the rate of 0.56 gram per meter of row, also delivers a high rate of rhizobia. This peat granule (particle size between 0.35 and 1.18 mm) is applied through a granular applicator and placed in the furrow with the seeds. When applied at a rate between 5 and 30 kg/ha, depending on the row width, the granular form will provide significantly more rhizobia than a seed-applied application which supplies only 210 g of inoculant on the quantity of seeds planted in one hectare. This granular inoculant provided 15.8 nodules per plant, significantly more than seed-applied inoculants, and a 2535 kg/ha yield (Fig. 2). In-furrow inoculants , granular or liquid, may be more costly but under many conditions will be superior in promoting nodulation.

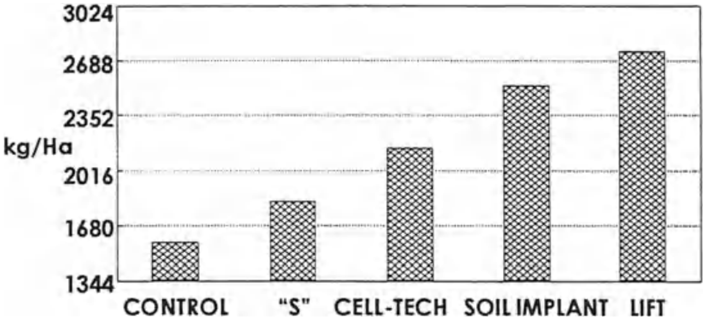

Figure 2. Soybean yield provided by no inoculation (control), non-sterile seed-applied peat powder ("S"), liquid on-seed (*Cell-Tech*), peat granule in-furrow (*Soil Implant*), and liquid in-furrow (LIFT).

The grower's choice of inoculant depends on the general method of planting, whether that legume has previously been grown on that land, equipment used for planting, acceptable product cost, and convenience. Preinoculation is the practice of applying the inoculant to the seeds by a seed processing company up to several months before planting, mainly on forage legumes such as alfalfa (*Medicago sativa*). This provides the ultimate in convenience for the grower who buys the seeds already inoculated. Seed pelleting is one form of preinoculation in which the peat inoculant is applied as a slurry utilizing a sticker (such as gum arabic) and the inoculated seeds are covered with finely-ground calcium carbonate (Brockwell, 1977).

Nitragin® Gold (LiphaTech, Inc., Milwaukee, WI) is a clay-based preinoculant. Cultures are mixed with the clays, dried and ground into a fine powder. Seed processors apply the dry product to the seed while mixing to provide a uniform application. Preinoculant on the seed generally has a 12- to 24-month expiration date. In the author's work, *Nitragin Gold* has an A_w of 0.400 to 0.650, mean particle size of less than 40 microns, viable rhizobia populations from 6 x 10^8 to 2 x 10^9 cfu/g, and excellent longevity over a 24-month period (Fig.3).

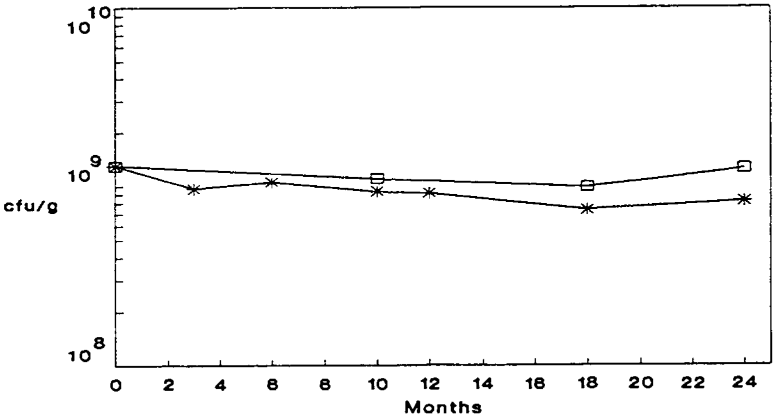

Figure 3. Longevity of *Rhizobium meliloti* formulated as *Nitragin Gold* and stored at 5°C (-o-) and 24°C (-*-).

Population estimates are higher with dried products when an initial slow rehydration is utilized to minimize rehydration upshock before continuing the dilution sequence (Kosanke et al. 1992). Bulk powder samples of *Nitragin Gold* were slowly rehydrated to 0.992 water activity by the gradual addition of diluent followed by a 10-minute period for moisture equilibration. Dried bulk powder formulations consistently provided a larger estimate after slow rehydration, with the samples averaging 6.5 x 10^8 cfu/g more than when rapidly rehydrated. A second slow rehydration procedure for preinoculated seed employed an environmental chamber at 24°C with relative humidity greater than 80% and incubated the preinoculated seed for one hour to allow moisture absorption. This slow rehydration of preinoculated alfalfa seed provided a general increase in the recovery of viable rhizobia with an average increase of 1328 cfu/seed.

The initial slow rehydration procedures presented for both the bulk dried powder inoculant and preinoculated seeds, rather than the common practice of rapid rehydration during dilution plating, more closely resembles the slower rehydration that may take place when preinoculated seed is planted in the field.

Several inoculants are available which better meet the needs of the changing legume management system and are more efficient with the newer soybean planting equipment, while providing convenience and quality. Improved applied technologies (formulations and application methods) must continue to evolve to satisfy grower needs, and may be instrumental for the implementation of newer basic technology in a changing production system.

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DEVELOPMENT OF *RHIZOBIUM* AND *AZOSPIRILLUM* INOCULATNS WITH ENHANCED POTENTIAL FOR FIELD APPLICATION

THOMAS, J., PALANIAPPAN, SP., HOPPER, W. AND
NIRMALA, C.B.
Centre for Biotechnology, SPIC Science Foundation 110 Mount Road,
Guindy, Madras 600 032, India

There are hundreds of commercial production units in India for microbial inoculants; nearly 20 of these have installed capacity to produce three million packages or more per unit per annum. Yet inoculant production is constrained by the lack of microbial strain efficiency and quality. To develop suitable *Rhizobium* inoculants and devise agronomic practices, the performance of strains under different agroclimatic conditions was assessed using normal inoculation procedures or in conjunction with nodulation inducers such as flavonoids. *Azospirillum* is used extensively in Southern India as a microbial inoculant. As the role played by *Azospirillum* in enhancing crop growth and yield is not yet clearly understood, colonization and plant growth promoting aspects of *Azospirillum* are also being studied in our laboratory.

Assessment of *Rhizobium* inoculants

Serological methods such as fluorescent antibody, membrane filtered immunofluorescence and dot immunoblot assays were used to evaluate competitiveness of *Bradyrhizobium japonicum* USDA110 as well as a native isolate *Bradyrhizobium* SSF8 in laboratory and field experiments with soybean.

Five strains isolated from local black soils were tested for competitiveness against fluorescent antibody positive USDA110. FA positive USDA110 occupied 59 to 65 per cent of the nodules when tested against local isolates SSF5, SSF6, and SSF7 with inoculant population ratio 1:1 (USDA110:SSF strain). In comparison USDA110 nodule occupancy was only 41 per cent when tested with SSF8. Another FA positive strain (SSF4) occupied only 29 per cent nodules when tested against SSF8, indirectly demonstrating the latter's competitiveness.

These early results indicated that USDA110 and SSF8 are the most competitive among the strains tested. The relative competitiveness of USDA110 and SSF8 were assessed using the Dot Immuno Blot assay (Somasegaran, Hoben, 1994) using strain specific antisera. When inoculated at a population density of 1:1 (USDA110:SSF8) 60 per cent of nodules were

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occupied by SSF8. Even when the ratio was changed to 10:1 and 1:10, SSF8 occupied nearly 52 per cent and 60 per cent of the nodules respectively, indicating the superior competitiveness of the strain.

Field evaluation of inoculants

The strains USDA110 and SSF8 were then assessed for their field performance at Nannilam, Tamil Nadu during Kharif (June-Aug) season in 1993. Nodule occupancy results indicate that SSF8 was much more competitive than USDA110 (Table 1 ii). In general, nodule number, shoot dry weight and grain yield were higher in plots inoculated with SSF8. In these plots the level of applied fertilizer N did not influence grain yield whereas N at 15 kg.ha^{-1} (75 per cent of farmers' practice) gave lower yield with USDA110 possibly due to the impact of high soil- and fertilizer-N on nodule derived nitrogen for plant metabolism. Significantly application of 20 kg N.ha^{-1} did not compensate the low yield in uninoculated plots.

Table 1 Field evaluation of inoculant strains for soybean. i) Nodulation, growth and yield

Treatment	Nodule No.Pl^{-1}		① Nodule Dry Wt		② Shoot Dry Wt		③ Grain Yield	
	USDA110	SSF8	USDA110	SSF8	USDA110	SSF8	USDA110	SSF8
I_0 N_0	8		90		5.30		1445	
I_+ N_0	20	23	284	221	9.31	9.36	1920	1712
I_+ N_{25}	23	28	330	271	8.81	9.33	1855	1925
I_+ N_{50}	21	25	340	207	8.47	8.94	1800	1990
I_+ N_{75}	18	23	259	204	7.53	8.86	1697	1922
I_0 N_{100}	4		28		7.02		1640	
LSD α=5%	3		44		1.26		190	

① mg.Pl^{-1} ② g.Pl^{-1} ③ kg.ha^{-1}; I_0 - No inoculation; I_+ -Inoculated

Mean of six replications; plot size 40 sq.m

N_0,N_{25},N_{50},N_{75} & N_{100} - N fertilizer % of normal farmers' practice. (N_{100} eq. to 20 kg N.ha^{-1})

ii) Nodule occupancy

Inoculant Strain ④	% Nodule formed by			
	USDA110	SSF8	Both	Neither
USDA110 alone	88 ± 8	16 ± 13	14 ± 10	10 ± 6
SSF8 alone	6 ± 5	92 ± 4	----	2 ± 4
Both (1:1)	18 ± 27	78 ± 7	6 ± 5	10 ± 6

④ with N fertilizer 50 % of normal farmers' practice.

The results from subsequent trials in 1993-94 with soybean and one with groundnut suggest the influence of soil N and soil pH on inoculant performance. At one site (near Cuddalore, Tamil Nadu) with high soil pH (8.0) and soil N (455 kg N.ha^{-1}) where native rhizobia were absent, the grain yield difference was not statistically significant although inoculation enhanced nodule number and nodule dry weight.

At another field (near Pondicherry) where soil N was low (225 kg N.ha^{-1}), pH 7.8 and native rhizobia absent, inoculation of USDA110 in plots given 15 kg N.ha^{-1} of fertilizer N, increased yield by 25 per cent. In the third trial (near Madras) where soil pH was 7.9 and soil nitrogen was low (285 kg N.ha^{-1}), USDA110 inoculation, with no addition of fertilizer N, increased yield marginally (6.6 per cent). Inoculation of SSF8, along with provision of 10 kg N.ha^{-1} fertilizer N, resulted in 17.3 per cent yield increase. The groundnut trial conducted at a field (Neyveli, Tamil Nadu) with soil pH 5.0, soil N of 262 kg N.ha^{-1} level and native rhizobia at 10^2 g^{-1} showed no significant difference in yield. Application of normal dose of fertilizer N (17 kg N.ha^{-1}) also had no effect on yield. To summarise, *Bradyrhizobium* SSF8 was more competitive, as determined by nodule occupancy, in almost all the experiments. High fertilizer as well as soil nitogen resulted in lower nodule occupancy especially in the case of USDA110. Plant dry weight and yield were comparable irrespective of the inoculant used.

Inducer flavonoids in chickpea

A crucial step for host specific nodulation is the induction of *nod* genes by specific flavonoids. Nodulation inducer molecules from chickpea, the second largest legume crop in India, were isolated from seed exudate. As *nod-lacZ* construct is not available in chickpea rhizobia, we have used the following bioassay. *Rhizobium* IC76 was grown in the presence of different fractions of seed exudate; the seedlings were inoculated with the cell-free culture filtrates and assessed for root hair deformation/curling activity. Using this bioassay four compounds having inducing activity were isolated by TLC and HPLC. One of the compounds was identified as biochanin A. Culture filtrate of chickpea rhizobia grown in the presence of biochanin A also induced Hac and Had activity in chickpea.

Nodulation enhancement by flavonoids

We reasoned that pretreatment of inoculant strains with host exudate fractions or the specific flavonoids will render the rhizobia more competitive in early legume - rhizobia interactions leading to better nodule occupancy and possibly yield.

Initially, *R. ciceri* exposed to ether extract of chickpea seed exudate was used as inoculant. The ether fraction was added to *R. ciceri* grown in Erlenmeyer flasks. The pre-exposed bacterium was injected to vermiculite packets and processed in the normal way prior to use as inoculant. Significant enhancement in nodulation was noticed with *R. ciceri* exposed to ether extract (72 per cent with *R. ciceri* IC76 and 112.5 per cent with *R. ciceri* F75; α=0.05) or whole seed exudate (36 per cent with IC76; α=0.05).

Alternatively ether fraction was injected along with *R. ciceri* and growth medium into vermiculite packets and the inoculant used subsequently on maturation; enhancement in nodulation was 100 per cent with IC76 (α=0.05) and 50 per cent with F75 (α=0.05). Significant enhancement in nodulation (27 per cent in both IC76 and F75; α=0.05) was also observed in another experiment where ether extract was added while coating chickpea seeds with vermiculite based inoculant.

To further substantiate the enhanced nodulation effect, pot studies were conducted with non-sterile garden soil. Chickpea plants inoculated with *R. ciceri* exposed during maturation in the carrier to ether extract of seed exudate or ethanol extract of seed powder or specific flavonoids (biochanin A, genistein and chrysin) exhibited highly significant enhancement in nodule number, fresh weight, dry weight and shoot dry weight over plants inoculated with unexposed rhizobia.

Table 2 Chickpea: Effect of preconditioning *R. ciceri* with nodulation inducers ①

Nodulation inducer	Nodule Fresh Wt (g) ②	Nodule Dry Wt (g) ②	Shoot Dry Wt (g) ②
None	**0.40** ± 0.18	**0.06** ± 0.02	**8.6** ± 4.5
Ether fraction	**8.77** ± 0.30	**1.05** ± 0.14	**24.8** ± 2.3
Ethanol fraction	**6.65** ± 2.05	**0.90** ± 0.33	**22.6** ± 4.3
Biochanin A	**3.31** ± 1.99	**0.59** ± 0.39	**18.0** ± 2.8
Chrysin	**2.24** ± 0.35	**0.33** ± 0.10	**14.8** ± 1.2
Genistein	**1.17** ± 0.57	**0.17** ± 0.07	**12.0** ± 2.1
Uninoculated	**0.81** ± 0.65	**0.12** ± 0.09	**11.2** ± 4.5
α	* * *	* * (0.0046)	* * *
LSD (0.05)	1.66	0.30	4.39

① Nodulation inducers were added along with inoculum while injecting into vermiculite packets; ② Nodule and shoot weight of six plants; mean of 5 replications; Ether Fraction - Ether fraction of the seed exudate; Ethanol Fraction - Ethanol extract of dry seed powder.

To evaluate the enhanced nodulation ability of preexposed rhizobia in field conditions, ***Rhizobium*** strain NC92 pre-exposed to the inducer compounds in the carrier was used to inoculate peanut seeds. Plants inoculated with pre-conditioned ***Rhizobium*** using seed exudate, showed increase in nodule number and grain yield compared to uninoculated plants or plants inoculated with unexposed ***Rhizobium***. Pre-exposure to the flavonoid genistein, significantly enhanced shoot dry weight and grain yield.

Table 3 Peanut: Effect of preconditioning *Rhizobium* NC92 with nodulation inducers ①

Nodulation Inducer	Shoot Dry Wt g / plant	Nodule Number / plant		Pod Number / plant	Pod Dry Wt g / 50 Plants
		TR	SR		
None	21.68 ± 0.94	31 ± 11	93 ± 65	10.7 ± 0.79	476 ± 58.51
Exudate	21.42 ± 3.75	35 ± 11	105 ± 87	10.5 ± 2.06	516 ± 46.60
Isoliquiritin	21.54 ± 1.61	22 ± 21	114 ± 59	9.5 ± 2.06	448 ± 60.74
Naringenin	20.26 ± 2.60	28 ± 13	84 ± 46	9.2 ± 2.06	496 ±103.00
Genistein	26.86 ± 2.55	34 ± 17	146 ± 70	11.5 ± 4.17	580 ± 99.11
Uninoculated	19.74 ± 3.75	30 ± 23	103 ± 23	9.1 ± 3.36	444 ± 111.28
α	* * (0.0019)	* (0488)		* * (0.0044)	
LSD (0.05)	3.14	14.75	64.5	2.7	99.79
LSD (0.01)	4.28				

① Nodulation inducers were added along with inoculum while injecting into vermiculite packets; TR - Tap root; SR - Secondary roots; Mean of five replications; plot size 20 sq.m

The trend in enhancement in nodulation leading to increased shoot biomass and grain yield by pre-conditioning rhizobia, with specific nodulation inducer flavonoids or specific host seed/root exudate preparations, was observed in field trials with peanut (4 trials) and soybean (1 trial). These studies suggest that rhizobia exposed to inducer flavonoids may increase nodulation and thereby enhance nitrogen fixation and crop yield.

Colonization of rice by GUS-tagged *Azospirillum*

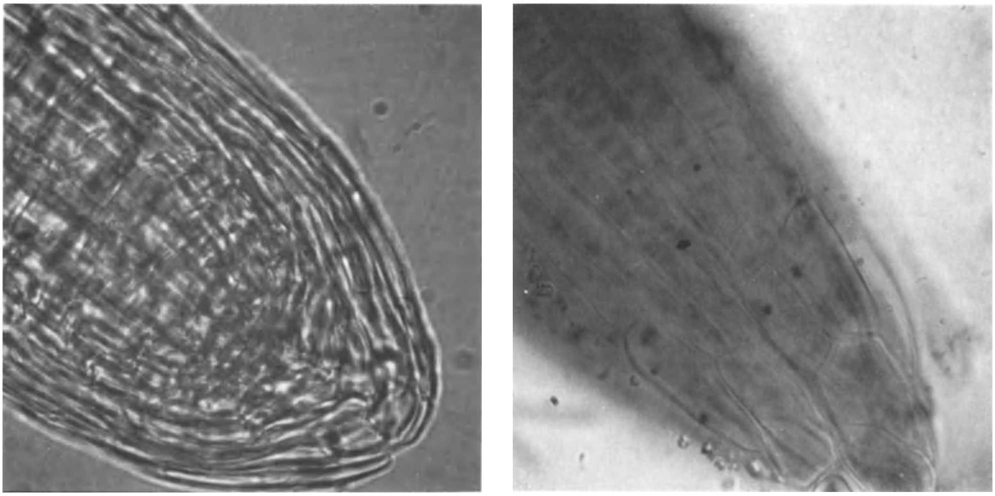
In contrast to *Rhizobium*, *Azospirillum* does not induce nodule formation in roots. Because of the lack of a clear phenotype, the mode of its entry and colonization in plant roots is not clear (Bashan *et al.*1991). The potential of reporter genes in studying the biology of plant associated bacteria was highlighted recently (Wilson *et al.* 1992). We used a transgenic marker system using *gusA* construct (mTn5SSgusA11, kindly provided by Kate Wilson, CAMBIA, Canberra) for the detection and monitoring of *Azospirillum* in association with rice plants.

Transconjugants containing GUS marker was identified by Southern blotting. Roots of seedlings inoculated with *Azospirillum* transconjugants containing 10^6 CFU.ml^{-1} were infiltrated with phosphate buffer containing 500 μg.ml^{-1} of the substrate X-Gluc (5Bromo-4-chloro-3-indolyl-β-D-glucuronide) and development of the blue colour was observed.

Growth of twelve transconjugants and wild strain, *Azospirillum* SL 33 was similar (10^8 CFU.ml^{-1} broth at the mid log phase and 10^6 CFU.ml^{-1} in Hoagland's solution 48h after inoculation). The transconjugants showed substantial GUS activity within 24h after substrate addition under free living and plant associated growth conditions. Uninoculated roots and roots inoculated with wild strain remained white indicating the absence of background activity in the assay. In nitrogen free growth medium, two days after inoculation, colonization was observed at the base of lateral roots implying the points of entry in the roots. Three to six days after inoculation, the whole root system stained blue but seventh day onwards, colonization was observed only in the tips of radicle (Fig.1), lateral roots and in the bulged tips of root hairs.

Fig 1 Histochemical localization of *Azospirillum brasilense* SL 33 labeled with *gus*A gene, in rice roots; Radical meristem of rice seedling inoculated with

SL 33 wild type *gus*A tagged SL 33



These observations were repeatedly recorded in subsequent experiments. Thus, after entry, bacteria gradually became localized to the lower parts of roots. This part corresponds to the microaerobic zone (Broek et al 1993) where azospirilla probably migrate to fix nitrogen. These initial results suggest that investigations using *gusA* tagged *Azospirillum* could prove valuable in understanding the symbiosis and in its field application.

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N_2-FIXING CYANOBACTERIA AS BTI TOXIN GENES DELIVERY SYSTEM - A BIOTECHNOGICAL APPROACH TO CONTROL MALARIA MOSQUITOES

BOUSSIBA S.[1], WU X.[1], PELEG N.[1], ZARITSKY A.[2]
(1) Microalgan Biotechnology Laboratory, Blaustein Institute for Desert Research, Ben-Gurion University at Sede-Boker, 84990, Israel.
(2) Department of Life Sciences, Ben-Gurion University of the Negev, P.O.Box 653, Be'er-Sheva 83105, Israel.

Rice is one of the most important crops in developing countries. About two thirds of the 143 million hectares of the world's riceland are used as wetland cultures: water from rainfall and irrigation sources is conserved to maintain shallow flooded conditions for almost the entire growth duration of the crop. Such water body creates ecological conditions favorable to the propagation of diseases such as malaria, schistosomiasis, and Japanese encephalitis. The malaria parasites of humans, which belong to the genus *Plasmodium*, are the most important in relation to rice field diseases. Malaria is transmitted by anopheline mosquitoes at a wide range of transmission levels as measured by the basic case reproduction rate. The anopheline mosquitoes, which carry malaria, and culicine mosquitoes, which carry many arboviruses asssociated with Japanese encephalitis, dengue and yellow fever, have larval stages that develop in water. In some area, rice fields facilitate anopheline breeding and may greatly increase transmission, also likely to prolong the transmission season (Bradley, 1988). The vectors (mosquitoes and the blackfly) require an aquatic environment at certain stages of their life cycle. Numerous studies have shown the association of wetland rice culture with mosquito-borne diseases in various parts of the tropical area. Most of the world's riceland farmers in tropical countries, east Asian and Latin American suffer from high incidence of diseases carried by mosquito larvae, which breed in water, as well as from lack of adequate nitrogen fertilizers for the rice plants at reasonable cost (Boussiba 1988, 1990, 1991). With application of a bifunctional transgenic cyanobacterial strain which will be released into the rice field for control of mosquito larvae and for excretion of nitrogen-fixed ammonium as a fertilizer while prevailing in rice fields longer than the commonly used laboratory strains, the life quality of the population in those area will be improved regarding these problems. The idea is realized by introducing the genes involved in the specific mosquitocidal δ -endotoxin formation from *Bacillus thuringiensis* subsp. *israelensis* (Bti) into an ammonium releasing mutant which we have derived from a rapidly growing rice field indigenous strain of *Anabaena* siamensis (Thomas et al., 1990). The high levels of mortality produced by several virulent strains *of B.thuringiensis* var. *israelensis* and *Bacillus sphaericus* in several culicine and anopheline larvae without undue effects on non target organisms, make them viable candidates for use in rice fields (de Barjac 1990). The high larvicidal activity of Bti is specific to water-dwelling dipteran species. The origin of the toxicity resides in δ -endotoxin contained in parasporal crystals produced during sporulation, which is composed of five major polypeptides (Hofte, Whiteley 1989) differing in their immunogenicity (Ibarra, 1986)

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and mosquito larvicidal potential. Synergism seems to exist between combination of the four (Ibarra, 1986; Chang, 1993).

The use of Bti is limited by economical constrains, due to low persistence under field conditions, in which it does not reproduce (de Barjac, 1990; Entwistle, 1993). The major reasons for the low efficacy of current Bti preparation are (a) Consumption by other organisms; (b) Sinking to the bottom of the water body; (c) Absorption to silt particles and organic matter; and (d) Inactivation by sunlight. Therefore it is imperative to develop less expensive biocontrol reagents, which are acceptable to farmers in developing countries. So far much effort is being expended around the world to solve these problems with attempts to isolate new mosquitocidal strains or species, to produce formulations of Bti with enhanced persistence in natural habitats of dipteran larvae, as well as to clone the genes responsible for toxicity into organisms which will express them coordinately and be ingested by the target organisms. Cyanobacteria which are abundant in diverse environment can be therefore candidate for this purpose. By introducing the Bti toxin gene operon into the ammonium-excreting mutant of *Anabaena siamensis*, it is hoped to constructed a bifunctional strain of ricefield indigenous cyanobacterium species with both capacities of supplying the ricefield with fixed nitrogen fertilizer and control of mosquito diseases. This genetically engineered species carrying Bti toxin genes will be released back to its original niches in the riceland ecosystems, there thy will be thriving and persistent for a longer time to perform the duties of mosquito vector control. Classical biological control, i.e. the importation and establishment of exotic natural enemies, is by far the most successful and promising way to replace the role of chemical pesticide for vector control. However some possible detrimental effects of imported natural enemies may cause environmental concerns and lead to serious legislative restrictions on classical biological control projects. The idea to introduce the engineered indigenous strain of cyanobacteria will certainly avoid this problem of disturbing the fine ecological balance with minimum side effects on the surrounding environment.

In the past few years, (De Marsac et al., 1987; Angsuthanasombat, Panyim, 1989; Chungjatupornchai, 1990; Murphy, Stevens Jr., 1992; Xu et al., 1993) positive data has been gathered supporting the idea that expression of the Bti δ-endotoxin genes in cyanobacteria, which proliferate near the water surface, may provide an alternative for prolonged delivery of Bti δ -endotoxin to feed mosquito larvae in the upper layer of aquatic habitats. But inthose works, there were still at least two technical problems needing to be solved. One of them is that the intact cells of unicellular cyanobacteria transformed with Bti δ-endotoxin gene did not show much toxicity to vector larvae except, when used as cell extracts or sonicated broken cells for toxicity assays. The other problem is that the transformed cells containing only a single toxin gene did not show satisfactory level of larvicidal activity even under the direct control of strong promoter PpsbA. The high toxicity of intact living cells is very important for practical application. So far only laboratory model species of filamentous nitrogen fixing cyanobacteria *Anabaena* 7120 and several naturally transformable unicellular strains have been used. No attempts have been performed yet to clone the Bti genes coding for the mosquito larvicidal polypeptides into a filamentous nitrogen-fixing cyanobacterial species prevailing in rice fields, which is ingected by mosquito larvae. This latter approach is currently being taken by our group in collaboration with colleagues from several developing countries. Biological control of malaria mosquitoes is the main goal of our project and the ricefield-isolated cyanobacteria, *Anabaena siamensis*, is considered as an appropriate shuttle organism to carry the Bti endotoxin genes. Reintroducing an indigenous but engineered strain into its atural habitat would contribute to the successful completion of this goal. An additional innovative aspect of this project is that it proposes, for the first time in the brief history of Bti, to construct an artificial operon out of the genes coding for toxicity and express them coordinately in the recipient organism. We plan to construct polygenic operons, consisting of varying combinations of five genes involved, under control of either LacP or T7P in *E.coli* (Zaritsky, 1990; Douek, 1992), or PpsbA in cyanobacteria and test their toxicity. All these combinations will be mounted on a *E.coli/A.siamensis* shuttle plasmid (Wolk, 1984) or suicide vector for sustaining and expression in *A.siamensis*. This goal will be acieved through the following specific objectives:

1) Mapping the large plasmid of Bti bearing the genes responsible for toxicity.

2) Cloning the genes involved in production of the δ -endotoxin from Bti in various combinations to form operons regulated ientically under PT7 expression vector in *E.coli*.

3) Testing the series of clones for their larvicidal activities.

4) Constructing the gene combinations in a proper shuttle vector for expression in *Anabaena* spp.

5) Developing an effecient transformation system for the ammonium-excreting *A.siamensis* mutant which had been derived in our laboratories.

6) Cloning the most effective constructed plasmids into this strain.

7) Characterizing the engineered strain by their mosquito larvicidal activity, ammonium excretion and growth properties.

8) Large scale production of the engineered strains in tubular bioreactor.

9) Controlled field trials. The final stage will be letthe engineered strains slowly released to the environment and subsequent assessment of its performance and far reaching effects on epidemic disease case occurring frequencies.

Specific objectives 1-4 have already been successfully achieved and will be published elsewhere. At this stage, it is conceivable that developing an efficient transformation system for the rice field isolate *A.siamensis* is a prerequisite of this project. But most cyanobacteria, except a few laboratory strains, are not suitable for DNA introduction by current available techniques. First, cyanobacteria have different endonucleases, sequence specific or unspecific, to degrade exogenous DNA, while their own DNA escapes digestion by methylation, by other unknown protection methods or simply by the absence of recognition sites (Tandeau de Marsac, Houmard, 1987). To modify plasmid DNA with methylation enzymes of a recipient cyanobacterium could greatly improve the transformation efficiency (Elhai, 1994) and therefore, it is imperative to characterize the endorestriction enzyme profile of *A.siamensis* and design a proper protection strategy against the restriction enzyme problem. Second, no wide host-range plasmid has been found in cyanobacteria. All of the constructed shuttle vectors can only replicate in one to several close species. So establishment of a gene-transfer system for a new isolated species can not rely on the available plasmids alone. Therefore, two questions need to be addressed before embarking on this project of constructing a bifunctional mosquito larvicidal strain of *A.siamensis*: a) What are the restriction barriers for transformation of *A.siamensis*. b) How to overcome the restriction barrier for establishing a transformation system of *A.siamensis*?

Endorestriction enzyme assay and plasmid protection by in vitro methylation.

Using a simple endorestriction enzyme assay (personal communication from Dr.Waards), several endorestriction enzyme activities have been detected. The first enzyme demonstrated was BamHI (Peleg, 1994). Others include PstI, HaeIII and probably some other non-specific nucleases. These three endorestriction enzymes were coicidentally found by Dr.Waards in the strain *Anabaena* ThaI (Houmard, Tandeau de Marsac, 1988), which has proved to be the same species as *A.siamensis*. Consequently, to conquer the restriction barrier, we have deleted the BamHI recognition site on the recombinant plasmids used for transformation or protected it by methylation. The BamHI methylase gene from pBamM2.2 has been incorporated into the suicide vector pRL 373 (to form pRL373 Bm) or into the helper plasmid used in conjugation. The recombinant pRL373 Bm carried a 2.2kb fragment with the BamHI methylase gene from pBam22.2 inserted through the HindIII site of the HetA 5' upstream region (Holland, Wolk, 1990). Plasmid protection was also carried out by commercial methylases M.BamHI, M.HaeIII, M.PstI and M.SssI. However, methylation by BamHI, PstI and HaeIII methylases was still not able to completely protect the plasmid pRL373 or λDNA against digestion by crude *A.siamensis* homogenates, but it was almost completely protected by additional methylase SssI protection. Electrotransformation of *A.siamensis* with the in vitro methylation-protected plasmid pRL373Bm showed that proper protection of the shuttle vector against the restriction barrier can improve the transformation efficiency 10-100 fold depending on the combination of methylases applied.

Construction of Cargo and Helper Plasmids Carrying Methylase Genes

A helper plasmid carrying the methylase genes of M.HaeIII and M.BamHI was constructed as described below. Similar approaches for construction of other helper plasmids carrying extra methylase genes such as M.PstI and M.SssI, are also under consideration. The 1.2kb EcoRI fragment of the M.HaeIII gene in PLJ HaeIIIM101-1 is subcloned into Bluescript sk$^+$. The recombinant plasmid is named pHMsk and screened out by X-gal color selection. An additional methylase of M.BamHi in a 2.2 HindIII fragment from pBamM2.2 is subcloned into pHMsk and results in pHBMsk. Then, a kanamycin-resistance cassette in a 1.1kb PstI fragment from pRL435K (a kind gift of Dr.Hirshberg) is inserted into the polylinker region of pHBMsk to form pHBM-skk. The 4.5kb PvuII fragment from pHBM-skk is then transferred into pRL542 at EcoRV site to produce the final helper plasmid pRL542HBM.

To maximize the stability of an introduced plasmid inside host cells distributed in the field under natural conditions without the selection pressure of antibiotics, it is best to use integrational plasmids, which are believed to be more stable than self-replicating plasmids. To introduce and maintain foreign DNA in *A.siamensis*, a few of suicide vectors have been constructed by standard recombinant DNA techniques (Sambrook, 1989). For cloning large fragment of *A.siamensis* genomic DNA, the EcoRI digested fragments of sizes ranging from 5-10kb are recovered from low melting point agarose gel after size fractionating EcoRI digected total genomic DNA by electrophoresis. The partial genomic library is constructed by cloning the fraction of fragments into suicide vector pRL447 (Elhai, Wolk, 1988) or pRL435K, and used in subsequent electroporation experiments (Chiang et al., 1992).

Restriction diminishes conjugal efficiency in cyanobacteria significantly (Wolk, 1984; Thiel, 1989). It has been shown that there are at least two restriction enzymes AvaI and AvaII present in *Anabaena* 7120. Preliminary results suggest that transfer efficiency is diminished by about one order of magnitude per unmodified AvaI site and by a lesser amount per unmodified AvaII site (Elhai, Wolk, 1988) Similar studies have been reported by others (Thiel, Poo, 1989; Moser et al., 1993). For electrotransformation of *Anabaena sp.* strain M131, a plasmid containing a single unmodified AvaII restriction site transformed cells with about 100-fold lower efficiency than did the same plasmid with a modified restriction site (Thiel, Poo, 1989) So restriction can significantly lower the transformation efficiencies of both conjugation and electroporation.

There are at least three endorestriction enzyme activities in *A.siamensis*, i.e., the isoshizomers of BamHI, PstI and HaeIII. The incomplete protection of shuttle vector and λDNA by in vitro methyletion with commercial methylases M.BamHI, M.PstI and M.HaeIII may indicate several possibilities: 1) there are more than three kinds of type II endorestriction activities inside cells of *A.siamensis*, or may be other kinds of restriction activities, such as nonspecific DNAase activities; 2) the methylation itself is incomplele, especially by M.HaeIII, a plenimena also found *in vivo* (Smith, Kelly, 1984); and 3) there are other sources of methylases with the same recognition sites may not able to protect the palsmids against the restriction activities from the host cells (personal communication eith Dr.B.Jack). It appears encouraging that with the additional methylation protection from methylase M.SssI, which recognizes 5'...CG...3' site, the shuttle vector and λDNA have been almost completely protected *in vitro*. Because M.SssI shows a wide spectrum of protection sites, it may be very useful in the application of transformation to a new species with an unknown restriction activity profile.

We wish to thank Prof. Wolk and Dr. Elhai for the supply of plasmids pRL542 and pRL373 and very helpful communications. We thank Drs.Brooks and Jack of New England Biolabs for the kind supply of plasmids pBamM2.2, pCAL-7, pGW4460 amd pLJHaeIIIM101-1. Our thanks also extend to Dr.Hirschberg for the kind gift of pRL435K.

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ROUND TABLE DISCUSSION: APPLICATION OF NITROGEN-FIXING MICRO-ORGANISMS IN THE ENVIRONMENT

AMARGER N.(1), AND BROUGHTON W.J.(2) *(Conveners)*
(1) Microbiologie des Sols, INRA, BV1540, 21034 Dijon, France
(2) L.B.M.P.S., Universite de Geneve, 1 ch. de l'Imperatrice, 1292 Chambesy/Geneve, Switzerland.

Importance of Biological Nitrogen Fixation. **R.Hardy** (USA) introduced the idea that sustainable agriculture is inconceivable without the replacement of synthetic N-fertiliser by biological nitrogen fixation (BNF). Foor this reason, continuing research and development in BNF is more justified than it ever was. Use of synthetic N-fertilisers has caused major perturbations of the global N-cycle including nitrate pollution of ground waters and production of green-house gases.

Transformations of Nitrogen. Inlarge measure, the efficiency of BNF depends on the plant genotype. Traditionally, ^{15}N technology has been used to determine the proportion of nitrogen derived from the soil versus the atmosphere. **D.Werner** (FRG) used these methods to measure nitrogen exchange in different systems. Denitrification was significantly higher in plots of nitrogen-fixing *Vicia faba* plots than in similar areas supporting *Lolium perrenne* and *Brassica oleracea*. Soil nitrogen levels also have a profound effect of the proportion of nitrogen fixed symbiotically. Using similar methods, **W.Merbach** (FRG) used different plants to show that in less effective symbioses, the amount of N absorbed from the soil is greater then in more efficient symbioses. **H.Bothe** (FRG) studied denitrifying bacteria in various soils. Denitrifying bacteria were mostly found in the vicinity of plant roots, whereas non-denitrifying bacteria were more abundant in bulk, plant-free soils. Olio-nucleotide promers were developed for dissimilatory nitrate, nitrite, and nitric oxide reductases. Their utility was shown with various *Azospirillum* species and they will be used for studies on the distribution of denitrifying bacteria in different ecosystems.

Inoculation Technology. **R.Smith** (USA) reported on promising formulations and application techniques for *Rhizobium* inoculants. Moist, peat-based granular formulations, or liquid preparations, are placed in the furrow with the seed ensuring high rates of applied bacteria, greater convenience for the grower, and separation of the bacteria from chemically treated seed. These techniques ensure higher populations of inoculant bacteria which may help overcome low competitivity of inoculants.

Lateral Transfer of Symbiotic Genes. *Lotus* species and their inoculants have been introduced into New Zealand soils. **C.Ronson** (NZ) found genotypically diverse strains of *R.loti* nodulating a stand of *L.corniculatus* that had been inoculated by a single strain of *R.loti*. Despite their diversity, all strains contained an identical symbiotic region to the original inoculant strain. This suggests that the symbiotic region (> 105 kb) was transferred to and integrated into the chromosome by horizontal transfer. In other words, lateral symbiotic gene transfer to non-symbiotic rhizobia may account for the evolution of new rhizobial strains in the field.

Survival and Persistence - Containment Systems. In order to limit genetic exchange among soil inhabitants, **F.O'Gara** (Ireland) has developed a containment system for *R.meliloti* based on a conditional lethal mutant. In this system, survival of the introduced strain can be made dependent on the continued presence of the host-legume, *Medicago sativa*. This way, it should be possible to control the life cycle of introduced rhizobial inoculants. In agronomic terms, this would address concerns of using very competitive rhizobial inoculants which would be difficult to replace in the future by new and improved strains. **F.de Bruijn** (USA) suggested that an alternative to address both the generally observed poor competitive ability of newly introduced (genetically modified) strains and their containment would be the "biased rhizosphere" system. This system would consist of a genetically engineered (crop) plant secreting the nutritional mediator rhizopine into the rhizosphere and beneficial soil microbes carrying the rhizopine catabolism gene cassette. The latter would be expected to gain a competitive advantage in the rhizosphere of the specific rhizopine producing plant and removal of the plants at the end of the growing season would then end this advantage, resulting in an opportunity for controlled containment.

Competition Amongst Introduced Organisms. Molecular methods have been developed to identify inoculant strains in nodules/soil. **S.Selenska-Pobell** (FRG) has identified a 130 bp region at the 5' and of the 23 S sub-unit of ribosomal DNA in 96 strains of the *Rhizobiaceae*. Two variable regions were found from which oligonucleotide probes might be developed for detection of rhizobia in soil, inoculants, and nodules. **M.Sadowsky** (USA) used rep-PCR fingerprinting to study competition for nodulation among genetically related *Bradyrhizobium japonicum* strains. Strains within the same sero-group compete fiercely witheach other for nodulation of *Glycine max*. Thus, intra sero-group competition may be more important, and exceed that found with serologically disparate strains.

Molecular Methods. Strain-specific PCR probes have also been developed for identifying rhizobia in competition. **K.Lindstrom** (Finland) used both species- and strain-specific PCR-probes to verify more traditional methods (e.g. antibiotic resistance) of evaluating competition. In experiments in which both effective and ineffective *R.galegae* strains were used to inoculate *Galega orientalis*, as few as 102 ineffective rhizobia per gram of soil could significantly reduce the nodulation success of the effective strain in yield terms, **K.Vlassak** (Belgium) studied some determinants of broad host-range and competition in *R.tropici*. Superior strains were introduced into the field to evaluate their competitive ability in nodule occupancy and persistence in three *Phaseolus vulgaris* cultivars. Unfortunately, after three years in the field, only 20% of the nodules contained the inoculant strains.

Degradation of Toxins. Soils contaminated with atrizine are common in Greece. **C.Douka** (Greece) isolated *R.meliloti* strains from soil cores taken at 5.5 m (depth of the water table). Some of the strains were able to degrade atrizine.

THE USE OF rep-PCR DNA FINGERPRINTING TO EXAMINE COMPETITION FOR NODULATION AMONG GENETICALLY-RELATED *BRADYRHIZOBIUM JAPONICUM*

SADOWSKY, M.J. and MOAWAD, H.A.
Department of Soil, Water, and Climate, and Department of Microbiology,
University of Minnesota, St.Paul, MN 55108 (USA)

A major emphasis of recent dinitrogen fixation research has been the development of genetic engineering techniques for the construction of rhizobial strains with enhanced nitrogen fixation. However, establishing these strains in a significant proportion of the nodules of a field-grown legume has been, and remains, a critical problem. Faced with competition from indigenous rhizobial and bradyrhizobial populations, even highly efficient N_2 fixing strains are destined to form few nodules and have a minimal effect upon N_2 fixation. Most studies on competitions for nodulation use genetically dissimilar and serologically distinct strains. These studies, however, do not reflect ecologically significant interactions among microbial populations. In nature, competition for nodulation among rhizobia is most severe among genetically related populations. Until recently, it has been impossible to examine competition for nodulation among near isogenic rhizobia due to technical problems in distinguishing one strain from another. Several molecular methods have been used to characterize closely related *Rhizobium* and *Bradyrhizobium* strains. Recently, it had been shown that the use of DNA primers corresponding to repetitive (rep) sequences, coupled with the PCR technique (rep-PCR), can be used to fingerprint the genomes of *B.japonicum* and *B.elkanii* strains. We have determined that the rep-PCR technique provides an effective means of differentiating between genetically-related and serologically indistinguishable *Bradyrhizobium* strains.

We have investigated intraserogroups competition for nodulation of soybean by *B.japonicum* serogroup 123 strains using the rep-PCR technique. Equal concentration, paired, competition studies in Leonard jars were done with four members of *B.japonicum* serogroup 123 (USDA 123, MN 9, IA 23, and OH 6). In competition studies done with strains USDA 123 and OH 6, 83.3% of nodules were occupied by OH 6. The same strain was also more competitive than strains IA 23 and MN 9, where the percentage of nodule occupancy by OH 6 was 83.3 and 91.7% respectively. The least competitive strain in this study was MN 9, it failed to occupy any nodules in competition assays with either USDA 123 or IA 23A. Results of our studies indicate that strains within the same serogroup can fiercely compete with each other for the nodulation of soybean. We contend that intraserogroup competition may be more important and exceed that found with serologically disparate strains, since habitat preference and growth and persistence factors are most likely more similar among serologically-related bradyrhizobia.

FORMATION OF DINITROGEN FROM NITROUS OXIDE BY *ESCHERICHIA COLI*

KALDORF M, BOTHE H.
Botanisches Institut, Universität Köln, Gyrhofstraße 15,
D-50931 Köln, Germany

Escherichia coli is able to reduce nitrous oxide stoichiometrically to molecular nitrogen. The highest activity of 1.9 µmol * mg protein^{-1} * h^{-1} for N_2O-reduction by *E.coli* is comparable to that found for N_2-fixing bacteria and about 1/10 of the N_2O-reductase activity of denitrifiers. When anaerobically grown *E.coli* K12 is incubated in the presence of high concentrations of $^{15}N_2O$ (5% v/v), small, but distinct quantities of $^{15}N_2$ can be detected as product of the N_2O-reduction by combined gas chromatography/mass spectroscopy. In contrast, denitrifying bacteria as *Azospirillum brasilense* Sp7 reduce $^{15}N_2O$ rapidly to $^{15}N_2$ by a dissimilatory N_2O-reductase.

The rate of N_2O-reduction by *E. coli* is much influenced by the growth conditions. Highest activities were obtained in cells grown anaerobically with nitrate as electron acceptor. The *fnr*$^-$ mutant JRG 861a is unable to perform N_2O-reduction indicating that the enzyme involved is under control of the FNR-system. *E. coli* is unable to grow anaerobically with N_2O as sole respiratory electron acceptor. In contrast to the dissimilatory N_2O-reductase of denitrifying bacteria, the N_2O-reducing enzyme of *E. coli* has a low affinity for N_2O (apparent K_m ~ 3.0 mM) and is affected by nitrite concentrations of less than 1 mM. Therefore N_2O-reduction might not occur under natural conditions in *E. coli*. N_2O-reduction has possibly no physiological role in *E.coli* but is the side reaction of an enzyme with another physiological role in intact cells.

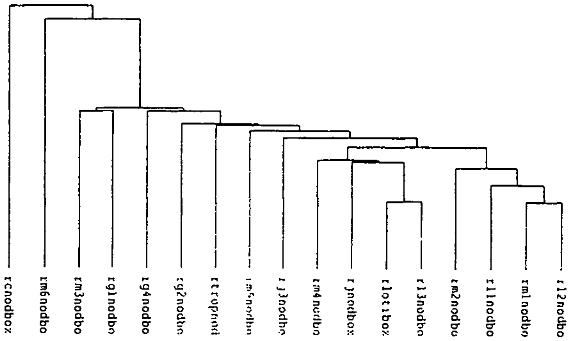
Dissimilatory N_2O-reductase is not found among the *Enterobacteriaceae*. Thus the only N_2O-reducing enzyme described hitherto for members of the *Enterobacteriaceae* is nitrogenase (in *Klebsiella pneumoniae*). It had already been established that *E. coli* has no nitrogenase. *E. coli* does not grow with N_2 as nitrogen source, and DNA-DNA-hybridization experiments with genomic DNA from *E. coli* and a *nif* H(D) probe from *Klebsiella pneumoniae* gave no hybridization signal. Thus *E. coli* possesses a further N_2O-reducing enzyme, the function of which remains to be identified.

Kaldorf M, Linne von Berg K-H, Bothe H (1993) Arch. Microbiol. 160, 432-439.

IDENTIFICATION OF NODULATION GENE CLUSTERS IN *RHIZOBIUM GALEGAE* STRAIN HAMBI 1174

LEENA SUOMINEN[1], GERMAN JURGENS[1], CHRISTOPHE ROOS[2],
LARS PAULIN [2] and KRISTINA LINDSTRÖM[1]
[1]Department of Applied Chemistry and Microbiology, P.O.Box 27, FIN-00014 University of Helsinki, [2]Institute of Biotechnology, P.O.Box 45a, FIN-00014, University of Helsinki, Finland

Galega sp. is a temperate legume which is nodulated only by *Rhizobium galegae*, a narrow host range rhizobial species described by Lindström (1989). The nodulation genes of *Rhizobium galegae* strain HAMBI 1174 have been cloned and identified in two separate clusters. The cosmid pRg30, sized 26 kb, contains common nodulation genes *nod*ABCIJ and a regulatory *nod*D1 gene, whereas pRg10, a 28 bp cosmid clone, carries a second *nod*D homolog and three of the four *nod*-boxes present in this strain. *R. galegae* belongs to the same phylogenetic branch with agrobacteria (see Lindström *et al.*, this volume). According to the sequence data and the protein alignments obtained from the *nod* gene region, the common nodulation genes *nod*A, B and C of *R. galegae* are closest related the corresponding genes of *R. leguminosarum* biovars *viciae* and *trifolii* and of *Sinorhizobium meliloti*.
A PileUp comparison shows the position of the four *R.galegae nod*-boxes within rhizobial *nod*-box sequences from different sources. Further analysis will reveal the relevance of this data in connection with rhizobial evolution.



PileUp sequence comparison of various nod-boxes found upstream from the nod operons.

RELATIONSHIP BETWEEN COMBINED NITROGEN AND SYMBIOTIC N_2 FIXATION OF LEGUMES

MERBACH, W.
Zentrum fur Agrarlandschafts- und Landnutzungsforschung (ZALF)
e. V. Muncheberg, Institut fur Okophysiologie der Primarproduktion,
Eberswalder Str. 84, 15374 Muncheberg, Deutschland

In each case mineral N applications were observed to have an inhibiting effect on symbiotic N_2 fixation already under way. Instead of fixed N, mineral N was taken up by the plants. However, the plants reacted to N fertilization by different growth in the various cereal legumes. In species with an efficient atmospheric N_2 fixation which lasts till the end of the growth stage (such as *Vicia faba* L. and *Lupinus luteus* L.) mineral N had no greater effect than an approximate compensation for the lack of fixed nitrogen and therefore had no influence on plant growth. In species where the root nodule symbiosis is less effective and terminates at an earlier stage (such as *Pisum sativum* L., *Lupinus albus* L. and *Glycine max*.(L.) Merr.) the amount of N absorbed form the mineral N fertilizer remains available for the plant as an additional N supply and can be used by it to enhance growth and crop yield.

N_2-FIXATION AND DENITRIFICATION IN FIELD PLOTS OF VICIA FABA

WERNER D and KILIAN S
Philipps-Universität, FB Biologie, Karl-von-Frisch-Straße, D-35032 Marburg, Germany

N_2-fixation in *Vicia faba* varies between 45 and 550 kg N_2 fixed per hectar and year (Roughley et al., 1983). The major part of this variation is due to physical and chemical soil factors, climate variation, plant cultivar, microbial and interaction variation. An other part is due to the different methods used to quantify N_2-fixation as the acetylene reduction assay, also in its modernized form, the N difference method, the xyleme solute technique, techniques utilizing enriched ^{15}N sources and the ^{15}N isotope dilution method, using the natural abundance of the ^{15}N isotope (Hardarson, 1994). The following data were established by use of a stable isotope spectrometer (VG SIRA Series 2 Instruments). With the last method denitrification was quantified as N_2O with the acetylene inhibition method (Kilian, 1994).

N_2-fixation rates of three cultivars of *Vicia faba*, using the non-nodulating cultivar cv. F48 as reference plant are summarized in Table 1. We see that for all plant organs (leaves, stems, pods and seeds) cultivar Albatross is superior to cultivar Condor and also to cultivar Piccolo on the same field.

Table 1. N_2-fixation with three cultivars of *Vicia faba* (cv. Albatross, Condor, Piccolo), reference plant: non-nodulating *Vicia faba* cv. F48, year 1992 (Kilian, 1994).

	kg N_2 fixed per ha and year				
Vicia faba cultivar	leaves	stems	pods	seeds	Total plants
Albatross	63.2	47.4	35.4	218.8	364.8
Condor	33.4	30.3	13.9	172.3	249.9
Piccolo	25.4	21.5	20.2	136.4	203.5

Denitrification rates were measured in field experiments for two years with plots of nodulated and non-nodulated faba beans, ryegrass and cabbage. Denitrification was significantly higher in plots of N_2-fixing *Vicia faba* than in plots of non-fixing plants like the non-nodulated *Vicia faba* mutant F48 and also *Lolium perenne* and *Brassica oleracea* (Fig. 1). The majority of this enhanced denitrification leads to the denitrification endproduct N_2 and not to N_2O in the filed. In plots of N_2-fixing faba beans an increase in soil nitrate of about 20 kg/ha was found between July and August. In plots of non-fixing plants soil nitrate decreased by about 10 kg/ha over the same period.

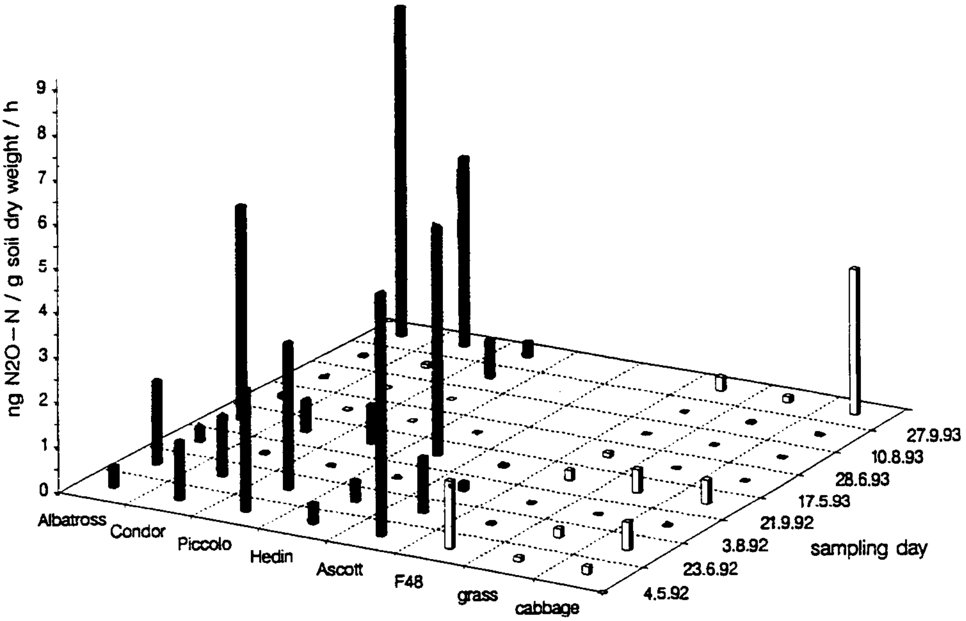


Fig. 1. N_2O-N production rates (1992 and 1993) shown separately for N_2-fixing *Vicia faba* cultivars (left) Albatross, Condor, Piccolo, Hedin and Ascott, the non-nodulating *Vicia faba* mutant F48, *Lolium perenne* and *Brassica oleracea* (right); N_2-fixing plants had higher rates than non-fixing plant ($P < 0.024$) (modified from Kilian and Werner, 1995).

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RELATIONSHIP BETWEEN BIOLOGICAL NITROGEN FIXATION AND HERBICIDE DEGRADATION

DOUKA, C.E. (1), DOSKARIS, J. (1), PROTOPAPADAKI, L. (1), VISANTINOPOULOS, S. (2) AND XENOULIS, A.C. (1)
(1) Institute of Biology NCSR "Demokritos" Agia Paraskevi 153 10, Greece.
(2) N.Ag.Re. F., 17 Egialias 151 25, Marousi Greece

The heavy worldwide use of herbicides has become a very serious threat to the Environment. Atrazine is the most widely used s-triazine herbicide. It can persist in soil environment for a long duration, with a half-life exceeding one year. Thus, atrazine contributes to environmental pollution affecting health standards. The aim of the study is to identify and examine a relationship existing between atrazine and the morphology- physiology of bacteria strains isolated from an extended soil profile down to the water level, among which special attention is given to *Rhizobium meliloti*. The identification of *Rhizobium meliloti* strains capable to degrade atrazine is of further extreme importance since such strains may find very efficient application in the environment.

From a wild area of Greece we took under sterile conditions soil samples, each a column 50 cm long, under a depth 5.5 m using a special drilling and sampling machine. From the 14 samples taken as above we isolated more than 200 strains of bacteria and about 50 strains of effective *Rhizobium meliloti*.

Preliminary Results:

1. During incubation of all non-sterile soils without addition of atrazine, the Total Viable Number of Bacteria increases while that of *Rhizobium meliloti* decreases.
2. During incubation with atrazine (0; 1; 2.5; 10; 20; 40; 60 and 100 ppm) the number of Viable Bacteria significantly decreases while the number of *Rhizobium meliloti* significantly increases. The above indicates that the *Rhizobium meliloti* present in soil samples under the examined conditions are benefited by atrazine. This sugests that it could be possible that they may utilize atrazine as a source of nitrogen and carbon giving hope that rhizobia strains may possibly degrade atrazine.
3. During incubation of non-sterile soil samples with atrazine a significant reduction in the concentrations of atrazine was determined while in the same sterile soils (gamma radiation 2.5 Mrad) no decrease in the amount of atrazine was seen.
4. Three pure *Rhizobium meliloti* strains isolated from the water level, after two months of incubation in sterile soil with 0; 20; 40; 60 and 100 ppm of atrazine demonstrated: a) survivability; b) their growth curves are similar; c) the above incubation does not affect at all the capability of the three strains to fix nitrogen.

All the above constitute a positive preliminary evidence that an atrazine- degrading bacterial system may actually exist in the examied samples. Furthermore, the present evidence does not exclude at all that this system may be associated with *Rhizobium meliloti* strains.

NITROGEN FIXATION IN PIGEONPEA-BASED INTERCROPPING ON ALFISOLS OF THE SEMI-ARID TROPICS

ADU-GYAMFI, J.J.[1], KATAYAMA, K.[2], ITO, O[3]., GAYATRI, D.[1], AND RAO, T.P.[1]
1) ICRISAT Asia Center, Patancheru 502 324, Andhra Pradesh, India
2) National Agriculture Research Center, Tsukuba, 305, Japan
3) National Inst. of Agro-Environmental Sci, Tsukuba, Japan

Though fertilizer N is often required if cereals grown on Alfisols are to achieve their yield potential, it is considered a risky investment because of the unpredictable weather, and limited and erratic rainfall. Opportunities for biological alternatives that can augment, and in some cases replace, N fertilizers must therefore be exploited. The inclusion of legumes in cropping systems, a common practice of resource-poor farmers in the Semi-Arid Tropics, is a cheap and efficient means of providing N-inputs to the system. We present here the results of experiments conducted in India, as a part of our on-going field studies, to examine different cereal/legume crop combinations and fertilizer management strategies to enhance N_2-fixation and N-use efficiency in Alfisols. Pigeonpea, sorghum, pearl millet, groundnut, and cowpea were grown either as sole crops or as intercrops. N derived from the atmosphere (N_{dfa}) and fertilizer (N_{dff}) were estimated by ^{15}N natural abundance and ^{15}N isotope dilution methods, respectively. Percentage N_{dfa} of pigeonpea was significantly increased when pigeonpea was intercropped with cereals than with legumes. The BNF by intercropped pigeonpea was enhanced when the percentage or the amount of both N_{dff} and N derived from soil (N_{dfs}) in pigeonpea were significantly lower than those of sole pigeonpea. The intercropped pigeonpea had less opportunity to acquire nitrogen from fertilizer and soil compared to sole pigeonpea, probably due to rapid depletion of N by the cereal component, which reduces available N concentration around pigeonpea roots and increases their dependency on BNF. The results suggest that more efficient utilization of nitrogen can be achieved by appropriate combination of component crops and their management.

RHIZOBIUM FREDII STRAINS ISOLATED AT VIETNAM.

BALATTI, P.A.(1), RODRIGUEZ-NAVARRO, D.N.(2), BUENDIA-CLAVERIA, A.M.(3), SANTAMARIA, C.(2), RUIZ-SAINZ, J.E.(3) and PUEPPKE, S.G.(1).
(1) Department of Plant Pathology, University of Missouri, Columbia, MO USA, (2) CIDA Las Torres-Tomejil, Apdo Oficial, Alcala del Rio, Sevilla, Spain, (3) Departamento Microbiologia, Universidad de Sevilla, 41080-Sevilla, Spain.

We have characterized SMX11 and SMH12, two of the fast-growing *Rhizobium* strains isolated by Pinochet and Cleyet-Marel from nodules of soybean plants growing in Vietnam. The objective of the work was to determine if these southeast Asian strains are indeed *R.fredii*, to define their relationships to conventional *R.fredii* strains from China, and to assess their potential for use as soybean inoculants. *R.fredii* strains USDA257 and HH103 were employed as standard reference strains.

All four strains are closely related, as determined by patterns of carbohydrate utilization, identical partial sequences of 16S rRNA genes, and RFLP analysis of genomic DNA with probes corresponding to *nodD*, *nodABC* and *nodSU*. Key differences were nevertheless detected by other methods. Strain HH103 is unique among the four strains in its ability to produce melanin in culture. Both Vietnamese strains are more salt-tolerant than the Chinese strains, and they are much more resistant to a spectrum of antibiotics, including nalidixic acid, tetracycline, kanamycin and spectinomycin. The most surprising distinction, however, is based on FAME analysis, which matched strain SMH12 to the *R.fredii* database but assigned strain SMX11 to *R.gallicum*, a newly descibed species of bean symbionts from France.

We tested the abilities of SMX11 and SMH12 to nodulate 41 legume species in replicated tests. Symbiotic phenotypes were identical on these species. Fix$^+$ nodules are produced on a variety of species other than soybean, including *Albizia lebbeck*, *Amorpha fruticosa*, *Cajanus cajan*, *Macroptilium atropurpureum*, *Macrotyloma axillare*, *Psophocarpus tetragonolobus* and several *Vigna* spp. *Phaseolus vulgaris* is nodulated by both strains, but nitrogen is not fixed.

Green house experiments in soil were conducted with the soybean cultivars Bragg, Davis, Nanking, Peking and Williams. The two Vietnamese strains are generally symbiotically superior to the Chinese strains, as assessed by nodule number and nodule dry weight per plant, as well as total plant dry weights. Cultivar specificity was evident. Nodule number on the cultivar Davis, for example, was ca. 10 times that on the standard cultivar Peking.

We conclude that the Vietnamese strains are related to conventional *R.fredii* and that they have potential for use as inoculants. We are currently attempting to determine the validity and significance of the FAME-based assignment of SMX11 to *R. gallicum*.

DNA FINGERPRINTING OF RHIZOBIA WITH THE USE OF DIFFERENT APPROACHES AND PROBES.

CHEMERIS A.V. (1), BAYMIEV Al.Kh. (1), SAFRONOVA V.I. (2), VAKHITOV V.A. (1)
(1) Department of Biochemistry and Cytochemistry, Ufa Scientific Centre RAS, Ufa, 450054, Russia, (2) Research Institute for Agricultural Microbiology, St.Petersburg - Pushkin, 189620, Russia

Such unique gift of Nature as fixation of atmospheric nitrogen by leguminous plants occuring in symbiosis with rhizobia, mankind must use with maximum efficiency. For this purpose a continuous search of new strains of symbiotic rhizobia, selection of known strains and also transposon mutagenesis and recombination is taking place. The group of bacteria fixing nitrogen in symbiosis with leguminous plants is rather large and diverse and nowadays is divided into several genera. Among these genera there is a number of species and biovars of rhizobia and also multiple strains with not clear classification. The method of fingerprinting with M13 phage DNA as probe was applied for several strains of *Rhizobium galegae* and *R.meliloti*. Cloned *Hae*III-fragment of protein III gene of M13 phage contain 15 bp hypervariable repeats was used in blot-hybridization as a probe. The use of restrictases of **GGCC**-family (*Hae*III, *Cfr*I, *BstZ*I, *Bsp120*I, *Msp20*I, *Eco147*I and *Not*I) and **GGNCC**-family (*Cfr13*I, *EcoO109*I and also *Eco47*I) in these experiments made it possible for us to reveal considerable RFLP which may be suitable for typing *Rhizobium* strains. The most interesting results were obtained after restriction by *Cfr13*I. The development of PCR methods opens new perspectives for genomic fingerprinting of symbiotic bacteria. On the basis of consensus sequence of hypervariable repeats of M13 phage, 15-mer primer **GAGGGTGGCGGTTCT** was synthesized for the use in RAPD analysis. The number of amplified fragments with this primer in genomes of different rhizobial strains varied from 1 to 10, and they were from 0.5 to 4.5 kb long. The use of a shorter arbitrary primer **TTACTGCGAACC** as a RAPD-marker with the same strains allowed to determine more complex DNA profiles. One of these strains was exclusively unique because only one band was revealed for the 12-mer arbitrary primers as well as for 15-mer primer homologous to hypervariable repeats of M13 phage. As tiny quantity of DNA is required for conducting PCR in contrast to blot-hybridization, it let us carry out RAPD analysis with this primer separately with symbiotic megaplasmid(s) and host DNA eluted from gel. The obtained results give evidence of preferential localization of these target sites for the arbitrary primer in host DNA.

TAXONOMY AND DISTRIBUTION OF RHIZOBIA INDIGENOUS TO SOUTH AFRICAN SOILS

DAGUTAT, HELGA & STEYN P.L.
Dept of Microbiology and Plant Pathology, University of Pretoria, 0002
Pretoria, South Africa

No comprehensive study of the indigenous South African rhizobia has been done, in spite of the richness of indigenous leguminous species and their possible practical importance in intensive as well as extensive, sustainable agriculture. The purpose of this study was to isolate as many as possible indigenous rhizobia, to identify them and possibly construct a taxonomy. We have collected 144 different nodulated legumes from diverse geographical regions and obtained 356 different isolates. We obtained rhizobia from *Cassia, Bauhinia* and *Schizolobium*, hitherto considered not to be nodulated. Our isolates were compared with 48 authentic rhizobial cultures using SDS-PAGE of soluble whole cell proteins. The resulting protein profiles were analysed by the GelCompar programme and a dendrogram was constructed. We found a large group of our isolates to cluster with members of the genera *Rhizobium, Bradyrhizobium* and *Sinorhizobium*. However, some of our results differed from published taxonomic data and we found possible new taxa not corresponding to any published taxa. Whereas *R.tropici* and *R.etli* are reportedly nodulating only species of *Phaseolus* and *Leucaena*, and *Phaseolus* respectively, we found *R.tropici* on *Bolusanthus* and *Spartium*, and *R.etli* on *Desmodium, Memolobium, Indigofera* and two tree legumes *Acacia melanoxylon* and *Chamaecrista stricta*. We found the tree legume isolates to form an assemblage of heterogeneous isolates and a plexus of lines and groups of various relationships. Isolates from different *Acacia* and *Chamaecrista* species mostly fall within the same clusters whereas *Sesbania* isolates show more variation. The rest of the tree isolates from six various genera (*Bauhinia, Albizia, Cassia, bolusanthus, Schizolobium* and *Millettia*) represent a phenotypically broad range of organisms. It seems obvious from our results that any comprehensive taxonomic exploration of the tree rhizobia will result in a proliferation of new taxa.

It is obvious that genetic studies are needed to further clarify the taxonomic and phylogenetic position of the new isolates and recognised genera of rhizobia.

EFFECT OF HOST GENOTYPE, AGROSTEMIN AND NITRAGIN ON NODULE NUMBER IN SOYBEAN

DUKIC, D., MANDIC, L., MARKOVIC, G., BOKAN, N., DUROVIC D.,
STEVOVIC V., IVANOVIC B.
Faculty of Agriculture, Cacak, Yugoslavia

The main objective of this paper is based on the fact that the legume species and cultivar genotypes as well as ecological adaptation of rhizobia strains influence the symbiotic activity and productivity. Therefore, the possibility of getting the maximum number of nodules in soybean by applying different doses of agrostemin and a standard dose of rhizobia inoculum (nitragin) (140-150 g/ha) is the main objective of this paper.

The following cultivar and lines were used: Kolubara, NS-L-96, NS-L-62, NS-L-110, NS-L-29 and NS-L-69. These were grown on smonitza which was treated with different doses of agrostemin (strong - 500 g/ha, weak - 250 g/ha) and the standard inoculum dose.

The data obtained indicate that there are significant differences among the lines studied as well as between cultivar Kolubara and the lines. The greatest effect of agrostemin dose and the inoculum was observed with the line NS-L-29 (50.00 nodules), followed by cultivar Kolubara (43.50) and the following lines: NS-L-62 (34.00), NS-L-69, NS-L-96 (29.50) and NS-L-110 (28.75). On the other hand, the application of the weak agrostemin dose and the inoculum resulted in the greatest number of nodules with cultivar Kolubara (42.25), followed by NS-L-110 (27.00), NS-L-69 (24.75), NS-L-29 (13.00), NS-L-96 (11.00) and NS-L-62 (8.75).

The results obtainded show that there is a good correlation between the dose of agrostemin and the number of nodules in cultivar Kolubara and all other studied lines. However, the effect of agrostemin and inoculum was not observed in some cases (as in NS-L-96, NS-L-62 and NS-L-29). It was recorded, namely, that the most of the nodules in the roots of legumes were created by spontaneous rhizobia found in the soil, and not by inoculated rhizobia.

Finally, it should be noted that the problem concerning the number of nodule bacteria in soil, which would provide the full realization of interaction with host plant, has not been solved yet, although it was the main objective of this paper.

MOLECULAR TECHNIQUES FOR DETECTION AND IDENTIFICATION OF RHIZOBIA IN SOILS, INOCULANTS AND NODULE SAMPLES

EVGUENIEVNA-HACKENBERG, E., SELENSKA-POBELL, S.
Department of Genetics, University of Bayreuth, D-95440, Bayreuth Germany

A region with high intra- and interspecies length variability was found at the 5' end of the 23S rDNA gene in 96 strains analysed, which belong to diffrent *Rhizobium*, *Bradyrhizobium* and *Agrobacterium* speies. This variability is caused by the presence of intervening sequences which lead to a fragmentation of the large subunit rRNA. Instead of 23S rRNA, two new rRNA species with sizes of approximately 130 bp (corresponding to the 5' end of the 23S rDNA) and 2.6 kb rRNA are present . In addition, in most strains of *R.leguminosarum* and *R.etli*, and in most of the *Agrobacterium* representatives, the 2.6 kb rRNA was fragmented to different extents in the central part. This central fragmentation is not caused by the presence of large intervening sequences. However, intraspecies variability in this region could be caused by short (10-20 bp) sequences (de Lanversin, Jacq, 1989).

The diversity in a group of six isolates from nodules of *Hedysarum coronarium*, referred to as *Rhizobium "hedysari"* (Squartini et al.,1993), was investigated by genomic PCR fingerprinting using three different arbitrary primers (Selenska-Pobell et al., 1995) and single primers corresponding to the bacterial repetitive BOX, ERIC and REP (Lows et al., 1994). By both arbitrary (AP-) and repetitive rep-PCR, an identification of every partucular strain was possible. In addition, in the dendrogram derived from the PCR patterns, the *R. "hedysari"* strains formed a stable cluster and the outgroup strains of *R. galegae*, *R. meliloti*, *R. leguminosarum* and *A. tumefaciens* were placed in outside positions.

The results obtained by the analysis of the AP- and rep-PCR fingerprinting were in taxonomical agreement to the RFLP analysis of PCR amplified parts of rDNA. PCR products corresponding to the spacer region between the 16S and 23S rDNA (IGS), to the 5'-half, and to the 3'-half of the 23S rDNA were digested with ten frequently cutting restriction nucleases - AluI, AvaII, CfoI, DdeI, HaeIII, HinfI, MspI, NdeII, RsaI and TaqI. In the resulting dendogram, the six *R. "hedysari"* strains clustered tightly together. Strain differentiation was achieved by digestion of the IGS or the 5'-half of the 23S rDNA amplificates with the two endonucleases MspI and HaeIII. the RFLP fingerprinting of the amplified IGS region was performed also using total DNA isolated from plant nodules or adding directly to the PCR reaction intact bacteria cells, isolated from nodules.

The taxonomical and discriminatory power of the rDNA-RFLP analysis and of the genomic PCR fingerprinting were compared by analysis of the already studied *R."hedysari"* strain A6 and the two additional isolates A10 and RH19. The RFLP patterns of the amplified IGS and 5'-part of the 23S rDNA generated by five endonucleases were identical. The ERIC- and REP- PCR fingerprints of the strain A6 and A10 were also identical and their BOX-PCR fingerprints were very similar. The rep-PCR fingerprints of the strain RH19 were more individual but still closely related to those of the strains A6 and A10. The three strains were clearly discriminated by AP-PCR analysis.

On the basis of the results presented here, a simple and highly reliable procedure for fast identification and taxonomical analysis of new rhizobial isolates is proposed. Fast screening, which can be performed using AP- and rep-PCR, enables one to demonstrate that new isolates belong to some of the previously established groups. Moreover, by AP-PCR, the specific characteristics of each strain can be scanned. On the other hand, using rDNA-RFLP-analysis, less complex patterns can be obtained, which are useful for the verification of the taxonomic characterization.

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SELECTION OF *RHIZOBIUM LEGUMINOSARUM* BV. *VICIAE* STRAINS FOR INOCULATION OF *PISUM SATIVUM* CULTIVARS.

FESENKO, A.N.
All-Russia Research Institute of Legumes and Groat Crops,
Orel, p/b Streletskoye, 303112, Russia

Proceeding from the analysis of 481 *R.leguminosarum* bv. *viciae* strains in pot and field experiments we demonstrated that the effective strains could be isolated from a rich medium-acid grey forest soil but not from a poor acid podzolic soil. It demonstrated that the contribution of the plant cultivars to the variation of shoot mass and N accumulation in pot experiments is higher, and contributions of cultivar-strain specifisity is lower than the contributions of strain genotypes. In field trials strain genotypes controlled rather strictly N percentage in grain (23,2% of total variation) and weakly grain mass (2% of variation). In contrast, the cultivar genotypes controlled strictly grain mass (25,7% of variation) but didn't control N percentage at all.

We identified ineffective strain 50 which can be used as a tester for assessing the nodulation competitiveness of the effective strains by an indirect and direct method (analysis of nodule appearance). We propose the scheme of accelerated selection of high-competitive *R.leguminosarum* strains out of soil using strain 50.

It was determined that increase of weight of shoots of different pea cultivars due to inoculation has negative correlation with absolute value of this trait of non-inoculated plants. On the other hand, value of yield increase of pea grain owing to inoculation has positive correlation with volume of yield in not inoculated variants ($r=+0,66$; $P <0,95$). It is possible that inoculation increases flow of assimilates for formation of yield of grain of high-productive genotypes of pea. This is displayed in considerable influence of genotype of rhizobia on yield of grain and number of beans per plant (genotype of bacteria controls 17,8% and 21,1% of total variation of these traits, correspondingly).

It was accertained that inoculation's greatest effect is displayed on defected (small or damaged) pea seeds. Maybe, inoculation is not only additional source of nitrogen nutrition but also "antistress" factor.

REACTION OF THE NODULATING PLANTS TO UREA NUTRITION

GLAZEWSKI S., KOCON A.
Department of Plant Physiology, Institute of Soil Science and Plant Cultivation, Pulawy, Poland

The effect of urea fertilization on the symbiotic nitrogen fixation, nitrogen absorption from the soil and fertilizer, dry matter and nitrogen accumulation in the vegetative organs and in the seeds of pea cv. Ramir was investigated in a pot experiment. Urea (0; 0.4; 0.8; 1.2 and 1.6 g N/8 kg of soil in 28 portions) was given to the soil or to the leaves respectively in the period of 20-48 days of plant vegetation.

Leafy treatment with urea inhibited N_2 fixation to a lower degree and stimulated N absorption from the soil fertilizer to a stronger one compared with the soil urea application. In the effect, after leafy treatment with urea, more total N was accumulated in the plant organs than after the soil urea treatment. The same was found for the vegetative dry matter accumulation. However, the yield of seeds was similar at both ways of urea treatment.

Tab. 1
Changes in the amounts of total N and dry matter yield in some elements of the pea-soil system, depending on 1 g of N fertilizer

Changes in:	N fertilization			
	to the soil		to the leaves	
	in g/pot	in percents of un-fertilized control	in g/pot	in percents of un-fertilized control
a) amounts of total N:				
- accumulated in plant-soil system	0.391	26.8	0.52	35.3
- fixed by host - Rhizobium system	-0.613	-42	-0.485	-33.2
- absorbed by plants from the soil and fertilizer	0.78	53.4	0.969	66.4
- accumulated in vegetative organs	0.099	6.8	0.28	19.2
- accumulated in seeds	0.067	4.6	0.199	13.6
- accumulated in the whole plants	0.166	11.4	0.479	32.8
- accumulated in the soil	0.225	15.4	0.036	2.5
b) dry matter of the plants :				
- accumulated in the vegetative organs	2.23	3.6	8.82	14
- accumulated in the seeds	5.57	8.9	5.67	9.1
- accumulated in the whole plant	7.8	12.5	14.49	23.1

FAO/IAEA COORDINATED RESEARCH PROGRAMMES ON BIOLOGICAL NITROGEN FIXATION

HARDARSON, G.
FAO/IAEA Agriculture and Biotechnology Laboratory, A-2444
Seibersdorf, Austria.

The Food and Agriculture Organization of the United Nations and the International Atomic Energy Agency have through their Joint FAO/IAEA Division in Vienna and the Agriculture Laboratory in Seibersdorf, Austria coordinated international programmes on biological nitrogen fixation (BNF) in developing countries for more than two decades. The main objectives of these programmes have been to enhance nitrogen fixation in various cropping systems and to develop and optimize the ^{15}N isotope dilution method to quantify N_2 fixation in leguminous crops. In connection to the various FAO/IAEA programmes fellowships and training courses are being offered.

Experiments, conducted as part of Coordinated Research Programmes (CRP), are performed in a number of countries simultaneously and, therefore, produce results applicable to a wide range of environmental conditions. Due to this replication of experiments, solutions to problems can often be found in one season. Another feature of the FAO/IAEA Coordinated Research Programmes is the transfer of research techniques to developing countries through the Research Co-ordination Meetings and workshops. Volunteer experts in the field of study help the FAO/IAEA staff with this task.

The BNF programmes initiated some twenty years ago focused on grain legumes and the development of ^{15}N methodology to measure BNF. These programmes were followed by others in which BNF in forage, pasture legumes, *Azolla* and tree legumes was quantified. The most recent programmes emphasize the enhancement of nitrogen fixation in some grain legume species through genetic improvements (Table 1).

The ^{15}N methodology has been found to be a powerful tool to measure biological nitrogen fixation in all the above systems. A review of the results has been published recently (Hardarson, 1994). Future programmes are likely to be on the use of leguminous crops in various cropping systems with emphasis on integrated plant nutrition management.

Table 1. Coordinated Research Programmes on biological nitrogen fixation which have been conducted by the FAO/IAEA.

Short title	Duration	Number of countries participating
Fertilization of grain leg.	1972-1977	14
Grain legumes	1979-1983	19
Multiple cropping	1980-1985	9
Pasture	1983-1988	19
Azolla	1984-1989	13
Bean in Latin America	1986-1991	7
Grain legumes in Asia	1987-1994	10
Tree legumes	1989-1997	15
Microbial ecology	1992-1996	12

When a CRP is initiated it is advertised in the Soils Newsletter, which is published regularly by the IAEA and sent cost free to subscribers. It is then possible for institutes in developing countries to send Project Proposals to the FAO/IAEA for consideration. Applications for Technical Cooperation Programmes, training courses or fellowships should be endorsed by and forwarded through the official established channels in each country.

For further information please write to: Soil Fertility, Irrigation and Crop Production Section, Joint FAO/IAEA Division, Wagramerstrasse 5, P.O.Box 100, A-1400 Vienna, AUSTRIA

Hardarson G (1994). International FAO/IAEA Programmes on Biological Nitrogen Fixation. In "Symbiotic Nitrogen Fixation", pp.189-202. Eds P H Graham, M J Sadowsky & C P Vance, Kluwer Academic Publishers.

MEASUREMENT OF NITROGEN TRANSFER BY PEA (*PISUM SATIVUM L.*) AND RED CLOVER (*TRIFOLIUM PRASENTE L.*) USING A ^{15}N LABELLING TECHNIQUE

HOOD R., WIDJAJANTO D.W.(1), SMITH K.A.
SAC, West Mains Road, Edinburg EH9 3JG, Scotland, The UK.
(1) Faculty of Animal Sciences, Diponegoro University, Hayam Wuruk 4A
Semarang 50224, Indonesia.

Direct transfer of nitrogen from legumes to non legumes is difficult to measure. A simple leaf labelling technique was developed to study the transfer. A single leaf of a legume is enclosed in small plastic bag containing highly enriched ^{15}N solution, for one week, this gives rise to an enriched legume and allows the transfer of N to be followed.

Pots of grass only, grass/red clover mixture and grass/pea mixture were planted. After 23 and 17 days respectively the red clover and pea plants were labelled. Soil and plant samples were taken at 8 and 21 days labelling.

Eight days after labelling the N content of grass shoot material, was significantly ($P<0.05$) higher in the grass only pots than in the grass clover mixture pots were not significantly different ($P<0.05$), yet significantly greater, nearly double that of the grass in the grass/pea mixture.

At the first sampling there were no significant difference ($P<0.05$) in the ^{15}N abundance of the grass grown in monoculture or mixtures. At the second sampling the ^{15}N abundance's of the roots of the grass grown with either clover or pea were significantly ($P<0.05$) higher than the grass grown in monoculture. Equivalent to an apparent transfer of 300 and 1114 µg from clover and pea respectively. There was a significant difference ($P<0.01$) in the shoot ^{15}N abundance of the grass only and grass/pea mixture. Suggesting that an average of 43 µg N (3.5% total N) was transferred to the grass shoots from the pea over 21 days.

These results suggest that N may be transferred from legumes to non legumes. However the route of transfer is still ambiguous and further research and development of this technique is required.

SUBDIVISION OF BRAZILIAN *Bradyrhizobium* STRAINS IN SPECIES *B. japonicum* AND *B. elkanii* AND CORRELATION WITH EFFICIENCY OF N_2 FIXATION AND COMPETITIVENESS.

HUNGRIA, M.[1].; NISHI, C.Y.M.[1]; BODDEY, L.H.[1] & VARGAS, M.A.T.[2]
[1]EMBRAPA-CNPSo, Cx. Postal 231, 86001-970, Londrina, PR, Brazil; [2]EMBRAPA-CPAC, Cx. Postal 700023, Planaltina, DF, Brazil.

Large scale soybean inoculation, in Brazil, started in 1960 and, since then, several strains were brought from other countries and introduced into Brazilian soils. We have studied some strains that were reisolated from areas that have traditionally been cultivated with soybean and that show higher competitiveness and efficiency of N_2 fixation than the parental rhizobia. Several morphological, physiological and genetic parameters were investigated, particularly the ones that differentiate the species *B. japonicum* and *B. elkanii*. Just one of the four strains recommended today in Brazilian inoculants, CPAC 7, can be classified as *B. japonicum* and this strain is a natural mutant of CB 1809, from CSIRO, Australia. Differences between these two strains included several profiles in RAPD analysis as well as higher competitiveness of CPAC 7, that is also able to produce more root hairs (Hai phenotype). Several strains isolated from savanna soils ("Cerrados"), belonging to the serogroup of SEMIA 566, a strain isolated in 1966 from a North American inoculant, were also studied. Contrarily to SEMIA 566, that shows typical properties of *B. elkanii*, the naturalized strains show intermediate characteristics among the two species, differing particularly in IAA and rhizobitoxine production, but none of the strains investigated was Hup^+. A savanna isolate, CPAC 15, is today recommended in Brazilian inoculants, and differences were found from the parental strain, SEMIA 566, in RAPD profiles, Hai phenotype and several physiological parameters. Strains belonging to *B. japonicum* species showed higher efficiency of N_2 fixation than *B. elkanii*. However, the most competitive strains were classified in a mixed genotype between the two species. Increases in soybean yield due to inoculation were observed even in soils with 10^6 cells of *Bradyrhizobium*/g of soil.

RAPID N_2 FIXATON DURING SOYBEAN POD FILL INCREASES PLANT BIOMASS AND SEED NITROGEN

IMSANDE, J.
Dept. of Agronomy, Iowa State University, Ames, IA 50011, USA

The N concentration of a soybean seed frequently is greater than 6%. Because of high seed-N demand a soybean plant may become N-deficient during pod fill. Also, it has been suggested that rapid N_2 fixation, which consumes 10 to 15% of the plant's net photosynthetic output, might decrease total plant biomass (Mahon,1983). Thus, we tested the relative effectiveness of N-source (i.e., potassium nitrate, ammonium nitrate, urea, or N_2 fixation, and combinations thereof), during pod fill, on the accumulation of both seed N and total plant biomass. Plants were grown hydroponically so that N source could be carefully controlled and total plant biomass could be accurately measured.

The amount of N assimilated during pod fill by plants fed only nitrate-N during pod fill was approximately 35% less than than that for plants fed the same amount of N in the form of ammonium nitrate or urea. Likewise, biomass accumulation during pod fill for plants deriving N solely from nitrate during pod fill was approximately 20% less than that for plants fed the same amount of N in the form of ammonium nitrate or urea. Also, the harvested biomass of plants grown in the presence of a low level of nitrate and fixing 300 to 900 mg N_2 plant^{-1} during pod fill was significantly greater than the harvested biomass of plants fed only nitrate-N during pod fill. It is concluded that soybean plants deriving N solely from nitrate during pod fill are frequently N-starved at harvest. Likewise, it is concluded that rapid N_2 fixation (300 to 900 mg N plant^{-1}) enhances net photosynthetic output of soybean.

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SYMBIOTIC NITROGEN FIXATION IN NATIVE LEGUMES SUBJECTED TO ARTIFICIAL SHADE IN A NEOTROPICAL SAVANNA.

MARIA LUISA IZAGUIRRE MAYORAL (1), ANA I. VIVAS AND TAMARA OROPEZA(2).
(1) Laboratorio de Biotecnología y Virología Vegetal. Centro de Microbiología y Biología Celular. Instituto Venezolano de Investigaciones Científicas, Apartado postal 21827, Caracas 1020 A, Venezuela. (2) Universidad Experimental Simón Rodríguez, Caracas, Venezuela.

The present study was carried out in a *Trachypogon* savanna of Estación Experimental La Iguana, Venezuela. The soils were characterized by 90% sand, a pH of 5.3, 8.3% aluminium saturation and a low cation exchange capacity. At the begining of the dry season, three savanna areas of 20 m^2 each were covered with a black greenhouse net. The net was placed at 1.5 m above ground, with closed lateral sides to avoid the penetration of reflected sun light. Under the nets there was a 90% reduction in the light intensity. Nets were removed toward the end of the rainy season, and all native legume species growing unde shade were collected for further analysis. Plants were analyzed in terms of ureide, a-amino-N, nitrate, chlorphyll and non-structural carbohydrate contents. The relative abundance of ureides (RAU) in shoots was used as indicator of the symbiotic nitrogen fixation rate in the individual legume species. Vegetation under the nets included the following species. *Chamaecrista tetraphila*, *C. rotundifolia* (Caesalpiniaceae), *Aeschynomene brassiliana*, *Desmodium barbatum*, *D. mollicullum*, *Eriosema simplicifolium*, *Galactia jussieuana*, *Indigophera lespedezioides*, *Phaseolus diversifolius*, *Stylosanthes capitata*, *S. guianensis*, *Zornia curvata* (Fabaceae), *Mimosa camporum* and *M. dobilis* (Mimosaceae). All species, except *Desmodium mollicullum* were also found in the sun-exposed areas, and all individuals were nodulated. In the species subjected to shade, RAU values in *C. tetraphila*, *E. simplicifolium*, *I. lespedezioides*, *S. guianensis*, *Z. curvata*, *M. camporum* and *M. debilis* were significantly higher than those detected in the same species collected in the sun-exposed areas. Whereas, a decrease was observed in *D. barbatum*. The rest of the species did not show differences in RAU between sun and shaded conditions. The statistical analyses of data revealed a significantly decrease in the chlorophyll and starch content in leaves of native legumes growing under shade. No significant differences were detected in the sugar content in leaves and roots, as well as in the starch content in roots between sun-exposed and shaded native legumes. In the area under study, the native rhizobial populations underwent a 3 fold decrease in soils collected under the net when compared to values obtained in the sun-exposed areas as a result of the reduced number of native legumes detected under shade.

EFFECT OF FLOODING ON N_2-FIXATION BY THE ORINOCO FLOODED FOREST SPECIES *MIMOSA PELLITA*

JAMES E.K.[1], BARRIOS E.[2], CRAWFORD R.M.M.[1]
[1]Plant Sciences Laboratory, University of St Andrews, St Andrews
KY16 9TH, UK.
[2]Centro de Ecologia y Ciencias Ambientales, IVIC, Apdo. 21827,
Caracas 1020A, Venezuela.

The intense seasonality of rainfall and the consequent rise in the Orinoco water level means that forests alongside tributaries may be subjected to flooding of up to 12 m depth for 7 months. This results in the leaching of N from soils, an unbalanced N-budget and subsequent selection pressure for trees with the ability to fix N_2 (Barrios, Herrera, 1994). Nodulated legumes were the dominant tree types in the flooded forest ecosystem studied by Barrios and Herrera (1994) and were considered to make an important contribution to the N-balance of the ecosystem, despite the extreme flooding which would normally be detrimental to N_2-fixing nodules. We have studied the adaptations which allows one of these trees, *M.pellita*, to fix N_2 under flooding.

M.pellita seedlings nodulate and fix N_2 within 3 weeks of inoculation with rhizobial strain MAP3-5 and can grow rapidly; 2 m in height in 6 months, even when the nodulated roots are completely flooded. Adventitious roots formed on flooded stems within a week of flooding, and these became nodulated after rhizobial infection of loose, erupted epidermal cells (no root hairs were present). Within 3 weeks submerged nodules developed leghaemoglobin and nitrogenase activity, with bacteroids expressing nitrogenase Fe-protein. Nodules developed under flooding were covered in aerenchyma and this was continuous with lenticellular aeration pathways on roots and aerial parts of the stem. Nodules were typically indeterminate with a persistent meristem; the persistence of nodule meristems may allow nodules to remain dormant under adverse conditions such as prolonged anoxia, and to resume production of N_2-fixing cells after floodwater recedes.

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EVALUATION OF COMPETITION AND EFFECTIVENESS OF *RHIZOBIUM LEGUMINOSARUM* AND SEARCH OF PEA LINES WITH INCREASED NITROGEN FIXATION

KASHAEVA, S. R., SADYKOV, B. F.
Institute of Biology, Ufa Scientific Centre, Russian Academy of Sciencies, pr. Octyabrya, 69, Ufa, 450054, Russia

Six *Rhizobium leguminosarum* strains collected at the Ufa region were tested in field conditions to estimate the competitiveness and effectiveness on 3 varieties of *Pisum sativum*. For identification of these strains in the nodules of field-planted pea we revealed some cultural differences between our strains on YEM-agarized medium (mannitol was substituted by glucose), media with congo-red dye (25 ppm) and media with tripheniltetrasolium chloride -TTC (20 ppm). The strains that looked equally on the basic medium could be coloured or not coloured on basic medium +TTC or differently absorb the congo red dye.

The competitive ability determined by the cultural properties of nodule bacteria was confirmed by the data of serological analysis of rhizobia in model experiments. The work with replicator makes this method simple and convenient. The obtained data on competitiveness and effectiveness of inocuated strains in field conditions were compared. The close connection between strain competitiveness and plant yield was discovered by statistical analysis.

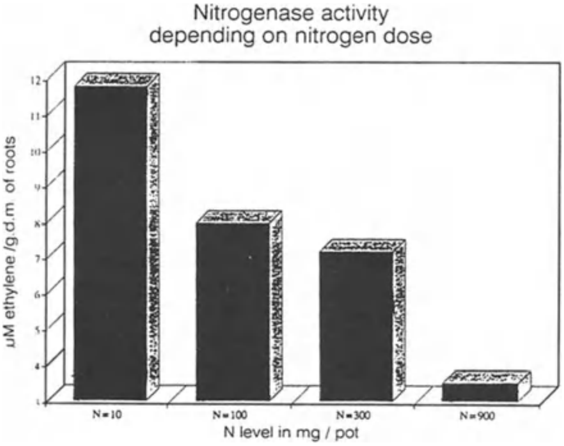
R. leguminosarum strains isolated from the hay-fodder variety of pea were demostrated to increase the total biomass of inoculated plants, and the strains isolated from food variety caused the rise of just the grain mass on hay-fodder variety of pea.This suggest the nessesity of parallel evolution of symbiotic partners in nature which must be taken into account at the choise of strains for inoculation.

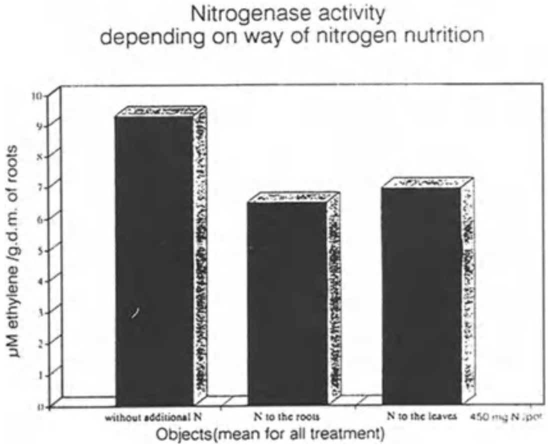
It was discovered also that within the modern pea varieties there are biotypes with different levels of simbiotic nitrogen fixation. A technique of isolating the biotypes combinating the increased (by 50%) level of nitrogen fixation and rised productivity was elaborated. The lines of pea with high level of symbiotic nitrogen fixation and seed productivity (in respect to the initial variety) were tested.

LEAFY N APPLICATION AND SYMBIOTIC N_2 FIXATION

KOCON A., MROCZKOWSKI W., GLAZEWSKI S., WOJCIESKA U., PODLESNA A.
Department of Plant Physiology, Institute of Soil Science and Plant Cultivation, Pulawy, Poland

We investigated the effect of nitrogen nutrition of faba bean plants on the seed yield, the effect of additional N supply to the leaves or to the roots during plant vegetation on the nodule formation and the nitrogenase activity. The experiment was performed in pots filled with sand at different mineral N rates (10, 100, 300 and 900 mg/7.8 kg - 5 plants), supplied as ammonium nitrate, at the beginning of the plant growth. During the vegetation a part of plants of every nitrogen level was additionally treated with 450 mg N given as urea to the leaves or to the roots. Nitrogenase activity in the root nodules was measured as the amount of ethylene formed, as a result of acetylene reduction in terms given in figures. The biomass production was also determined. The basic nitrogen nutrition, with ammonium nitrate, as well as additional treatment with urea, given to the leaves or to the roots, depressed nodule formation and nitrogenase activity. Inhibitory effect of urea treatment on the nitrgenase activity was greater when urea was supplied to the roots than to the leaves. Independently of nitrogen supply the highest nitrogenase activity was measured at flowering stage.





THE ACTION OF RHIZOBIAL EXOPOLYSACCHARIDES ON RHIZOGENESIS AND STRUCTURE OF PEA ROOT NODULES

KORELOV V.E. (1), KHAILOVA G.F.(1), KOSENKO L.V.(2).
(1) Institute of Plant Physiology of Russian Academy of Sciences, Botanicheskaya 35, 127276 Moscow, Russia
(2) Institute of Microbiology and Virology of Ukrainian Academy of Sciences, Zabolotny St., 154, Kiev, 252143, Ukraine

The biological activity of exopolysaccharides (EPS) from *Rhizobium leguminosarum* bv. *viciae* were tested on pea plants. Pea seeds were sterilized and grown aseptically for three days. Then the seedlings were incubated with 0.4% EPS solution under aseptic conditions. Seedlings incubated with water were used as control plants. After EPS treatment plants were grown aseptically on agar medium or on water culture. Treatment with EPS from two highly virulent strains (97 and 248b) led to the activation of lateral root formation when plants were grown in aseptic conditions. The number of lateral roots exceeded the control treatment by 156 and 32% in effective strain 97 and uneffective strain 248b, respectively. We assume that rhizobial EPS stimulated root cell proliferation. Spontaneous nodulation was observed when plants were grown in water culture. The treatment of roots with EPS from effective strain 181 for 1 and 2 h caused ultrastructural alteration in root nodule symbiosomes. Peribacteroid space (PBS) in symbiosomes significantly decreased. We suggest that PBS decreases favors the nitrogen fixation by nodules we have already demonstrated that nodules lacking PBS (alfalfa nodules) exhibited the highest nitrogen fixing activity.

NITROGEN-FIXING ABILITY OF *LUPINUS ANGUSTIFOLIUS L.* ACCESSIONS UNDER INOCULATION WITH NODULE BACTERIA STRAINS.

KOZHEMYAKOV A.P.(1), KURLOVICH B.S.(2), EMELJANENKO T.A. (2).
(1) Research Institute for Agricultural Microbiology, Podbelsky Shosse 3, St.Petersburg - Pushkin 8, 189620, Russia
(2) N.I.Vavalov All-Russian Institute of Plant Industry, 42, B.Morskaya Str.,St.Petersburg, 190000, Russia.

The intensification of the role of biological nitrogen fixation is considerably determined by efficiency of bacterial preparations wich help to accumulate nitrogen and to increase legume plants yield. In Research Institute for Agricultural Microbiology there is the collection of nodule bacteria wich are used for inoculation of legume crops . In N.I.Vavilov Institute of Plant Industry stores a collection of plant genetic resources. The revealing of nodule bacteria strains with high activi ty and efficiency of symbiotic nitrogen fixation is of the great importance. Seven new nodule bacteria strains selected from different sources were tested on two *Lupinus angustifolius L.* varieties: Nemchinovsky 846 from Russia and Yandee from Australia. The *Rhizobium lupini* strains were: 1604, 1613, and 1607 from *L. angustifolius*, 1610 from *L. luteus*, 1630 and 1635 from *L. albus* and 1625 from *L. polyphyllus*. The seeds were inoculated with bacteria and the plants were growen in pots with sand. The inoculation gave considerable increasing effect to biomass of both varieties beginning from the stage of fruitification. However the strain influence differed depending on the stages of development. The highest green mass of plant of Nemchinovsky variety at the fruitification stage was under inoculation with 1635, 1604 and 1610 strains. But at the complete ripeness stage the highest green mass was under 1604, 1630, 1635 strains. InYandee variety the highest green mass of plant at the stage of fruitification was under inoculation with 1604, 1607, 1630, 1625 strains and under 1630, 1604, 1613, 1625 strains at complete ripeness stage. The nitrogenase activity during the budding stage in Nemchinovsky variety was low but in Yandee variety it was higher. However their were no correlation between nitrogenase activity and plant biomass. The highest efficiency of symbiotic nitrogen fixation for the regionally adapted variety of lupine (Nemchinovsky 846) showed 1604, 1630, 1607 strains. The highest efficiency for Australian variety showed 1604 and 1630 strains of *R.lupini*.

PEA PLANT AS A TOOL FOR THE SEARCH A SINGLE STRAIN IN SOIL.

KULIKOVA, O.A.(1), USHAKOV, K.V.(1), NAUMKINA, T.S.(2), TIKHONOVICH, I.A.(1).
(1) Research Institute for Agricultural Microbiology, St.-Petersburg-Pushkin, 8, 189620, Russia.
(2) Research Institute of Grain and Legume Crops, Orel, 303112, Russia.

Strain *Rhizobium leguminosarum* bv. *viciae* A-1 isolated from North-West of Russia is highly effective and capable of nodulating Afghan peas (Chetkova, Tikhonovich, 1986). The plant resistance is controlled by a single recessive gene *sym2 (*Lie, 1984*)*. The ability of bacterial strains to nodulate Afghan peas is due to an extra gene named *nodX* and firstly described in strain TOM (Davis et al., 1988). Near isogenic lines (NIL) varied in *sym2* allele were obtained by crosses of European line NGB1238 X Afghan line NGB2150. NGB1238*sym2sym2* is absolutely resistant to normal bacterial strains.

The specificity between *sym2* and *nodX* together with apparent low frequency with which nodX is encountered in naturally occuring bacterial isolates suggested that this specificity could be exploited to prevent indigenous bacterial strains and allow inoculant strain to nodulate and establish a symbiotic association.

Of great interest for us is to estimate how stable genetically is the strain A-1 in soil and the possibility for application of such highly complementary symbiotic pairs in agricultural practice. In greenhouse experiment the bacterial isolates from nodules formed by A-1 were tested on resistance to streptomycin and rifampicin and by their immunological cross-reactivity with the antibodies against cells of strain A-1. Then these isolates were analysed by hybridization of their total and EcoRI digested DNA with ^{32}P -labeled DNA probe containing *nodX* of strain A-1. We have discovered 3 bacterial isolates which do not give positive signal for the presence of nodX but retain the ability to nodulate Afgan peas. The field trials were conducted in Central Russia during 1992-1994. Afghan line NGB2150 was inoculated by strain A-1 in 1992. The plants of this line without inoculation were used as control. During the 2 subsequent years cv.Nord (1993) and NGB2150 (1994) were grown in the same sites of field without added inoculation. It turned out that some indigenous bacterial strains contain *nodX* in their genome. After last growing season the soil samples were collected and soil suspension was used as inoculum for NIL. The bacterial isolates from nodules were tested for the presence *nodX* and the composition of plasmids and lypopolisaccharides. Some genetic instability of strain A-1 after introduction in soil and interaction with host plants was observed.

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DIRECTIONS AND METHODS OF BREEDING GRAIN LEGUMES WITH HIGH NITROGEN-FIXING ABILITY

KURLOVICH, B. S.(1), KOZHEMYAKOV, A. P.(2),
EMELJANENKO, T. A.(1), KARTUZOVA, L.T.(1)
(1) N. I. Vavilov All-Russian Research Institute of Plant Industry, B. Morskaya str., 42, St. Petersburg, 190000, Russia.
(2) Research Institute for Agricultural Microbiology, Podbelsky
Shosse 3, St. Petersburg-Pushkin 8, 189620, Russia.

Main objectives are: 1) selection of effective nodule bacteria strains for inoculation of high productive varieties of legume crops; 2) breeding of varieties with high nodulation activity and efficiency of symbiotic nitrogen fixation during their interaction with corresponding nodule bacteria strains; 3) search for varieties able to select effective bacterial strain from soil; 4) breeding of plants responding positively to special selective strains (Kartuzova et al., 1991; Kozhemyakov et al., 1991). In the field experiments 28 *Lupinus angustifolius L.* genotypes of different geographical origin were tested under 4 nutritious backgrounds: 1) without any application; 2) under seeds inoculation with bacterial preparation rhizotorfin containing *Rhizobium lupini* strain 363a; 3) under mineral nitrogen application; 4) under mineral nitrogen fertilizer application and seeds inoculation with *R. lupini*. In most cases application of rhizotorfin increased plant productivity. The best efficiency showed the varieties: Ladny and Nemchinovsky 846 from Russia, Vada 10 from Byelorussia, Mirela and Emir from Poland. The application of rhizotorfin in Nemchinovsky variety increased the height from 20 up to 30 cm, the green weight from 4 up to 8 g, the number of root nodules from 13 up to 27 at the stage of budding-initial blossom.

Inoculation did not increase height and green weight of plants of Australian varieties Yandee, Jorrel and Unicrop. The low and negative effect of rhizotorfin application in lupine varieties from Australia and Palestine is connected with discrepancy bacteria strain in respect to the plant genotypes.

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NEW APPROACH TO MODELLING THE INITIAL STAGE OF A LEGUME-RHIZOBIAL SYMBIOSIS FORMATION

LAGUTINA,T.M., TIMOFEEVA S.V., VOROBYOV N.I .
All-Russia Research Institute for Agricultural Microbiology, Podbelsky
Shosse 3, St.Petersburg-Pushkin 8, 189620, Russia.

The membrane filter technique was used for modelling the nitrogen-fixing inoculant behaviour on the surface of roots.

According to this method the bacterial inoculum was placed on the membrane filter. Then the plant seed was introduced in the centre of the membrane filter too. The filter containing the bacterial inoculum and the seed was inserted in a nylon bag and introduced into soil. Vegetative development of the plant was normal in this model system during 15-20 days. The seed germinated and the plant roots rested on the membrane filter surface. The bacterium from the membrane filter colonised the surface of the roots. After an incubation period the membrane filters with the plant were taken out. The quantity of bacteria was determined on the membrane filter surface (rhizosphere) and on the roots (rhizoplane) by a plate dilution method. Thus this approach permitted to study the bacterial plant root colonisation and a survival of this bacteria in rhizosphere in dynamics.

Using this approach we investigated the influence of various nonsymbiotic nitrogenfixing bacteria - *Arthrobacter mysorens, Azospirillum lipoferum* on the behaviour of various *Rhizobium meliloti* strains in the zone of *Medicago sativa* roots. The inoculum of *Rhizobium meliloti* was placed together with nonsymbiotic bacteria and the *Medicago sativa* seed on the membrane filter. The amount of *Rh.meliloti* and nonsymbiotic bacteria was counted after the incubation in soil individually: on the membrane filter and on the root surface. This permitted to study an interaction of symbiotic bacteria with nonsymbiotic ones in rhizosphere and in rhizoplane in dynamics. Applying the cluster analysis it has been determined that the strain of *Rhizobium meliloti* controlled the survival of the symbiotic and nonsymbiotic bacteria in rhizosphere. The activity of root colonisation of *Rhizobium* was controlled by *Arthrobacter mysorens* or *Azospirillum lipoferum* with the bacterium *Arthrobacter mysorens* has more active influence than *Azospirillum lipoferum*.

SIGNIFICANCE OF SULPHUR NUTRITION FOR BIOLOGICAL N_2 FIXATION OF DIFFERENT LEGUMES

LANGE A., SCHERER H.W. and WERNER W.
Institute of Agricultural Chemistry, University of Bonn, Meckenheimer Allee 176, D-53115 Bonn, Germany.

Although it is well established that sulphur fertilization of legumes may increase yield and N content only a few experiments were conducted to investigate the influence of sulphur nutrition on microbiological N_2- fixation (BNF). For this reason on the aim of our investigation was to evaluate the effect of S nutrition on BNF.

In a pot experiment with *Medicago sativa, Trifolium pratense, Pisum sativum and Vicia faba minor* sulphur was applied in three increments (0.25 and 200 mg S pot^{-1}) in combination with 50 mg N and 1250 mg N pot^{-1} . N was supplied as ^{15}N labelled NH_4NO_3 (10 atom % ^{15}N excess). The substrate (10 kg pot^{-1}) was a mixture of quartz sand and the top soil of a luvisol derived from loess (2:1).

The following results were obtained:

- The high S application rate resulted in significant higher N accumulation , which is caused by a higher BNF. The percentage of BNF from total N accumulation was increased by S application from about 90 to 95%.
- The influence of S on the amount of BNF was less pronounced with the high N application rate.
- With the grain legumes the weight of nodules was increased by the high S application rate.
- The higher number of nodules per pot with the high S application rate is the result of a better root growth.
- Independent of the N supply dry matter yield was highest with the high S increment.

BACTERIAL IRON ACQUISITION IN THE SOYBEAN- *Bradyrhizobium japonicum* SYMBIOSIS

LE VIER, K.D. (1), DAY, D.A. (2), AND GUERINOT, M.L. (1)
(1) Department of Biological Sciences, Dartmouth College,
Hanover, NH 03755, USA. (2) Division of Biochemistry and
Molecular Biology, Australian National University, Canberra,
A.C.T.2601, Australia

We are interested in the role of iron in the relationship between soybean and its nitrogen-fixing endosymbiont, *Bradyrhizobium japonicum.* To this end, we are investigating the mechanisms by which *B. japonicum* acquire iron both as free-living bacteria and when they are associated with the soybean host.

In cultured cells of *B. japonicum*, certain outer membrane proteins are made only when cells are grown under iron-limiting conditions and are presumably involved in uptake of iron complexes. We have purified and microsequenced an iron-repressible outer membrane protein (IR-OMP) from cultured *B. japonicum* cells in order to clone the gene encoding this putative ferric-chelate receptor.
Characterization of the clone is in progress.

We have attemped to determine the form(s) of iron that can be transported across the peribacteroid membrane (PBM) and the bacteroid membranes (BM; outer and inner membranes) to supply the bacteria with the high levels of iron required for nitrogen fixation. Silicon oil transport assays using radiolabelled ferric-chelates have shown that ferric citrate is able to move across the PBM, but not across the BM, and that a number of other ferric chelates can cross both membrane systems. Pulse-chase experiments show that a significant amount of iron accumulates in the peribacteroid space (PBS) after crossing the PBM, suggesting that the PBS serves as an iron storage pool. These data support the idea that the PBS is analogous to the vacuolar space: both are acidic compartments, contain some common enzymes, and are involved in iron storage. We also investigated the mechanism involved in iron-chelate transport across the PBM to determine if transport is via a ferric-chelate reduction step such as that found in the root plasma membranes of many plants.

EFFECTS OF LIMING AND INOCULATION ON PEA YIELD

PETRI LEINONEN
Agricultural Research Centre
Partala Research Station for Ecological Agriculture
FIN-51900 Juva, Finland

In earlier experiments the response of inoculation on pea (*Pisum sativum*) yield in scandinavian field trials has been inconsistent. In most of the trials, indigenous population of *Rhizobium leguminosarum* biovar. *viciae* has not been analyzed. I organized a set of field experiments, where also the size of indigenous *Rhizobium* population nodulating peas was determined by MPN-technique. Size of the population was highly pH-dependent: in higher pH also more Rhizobia were found. Inoculation of pea seeds didn't therefore increase yield in soils having a pH above 5.8, whereas in acidic soils the inoculation increased yield considerably.

A question of the development of indigenous *Rhizobium* population still remains unanswered. Do acidic soils still contain efficient strains of *Rhizobium*, although not active in low pH conditions ? How does liming affect on *Rhizobium* population nodulating peas ?

Three soils with different origin and initial pH was selected for further studies. One of them has high pH (6.5) where inoculation did not increase yield in field trial, whereas with more acidic samples, inoculation of pea seeds with *Rhizobium* had contributed to higher yield. Soil samples were layered onto a growth mixture (peat + sand), which was limed either to initial soil pH or to pH 6.5, considered as optimal for pea growth. Thus chemical and physical conditions for plant growth in pots were similar, the only difference was the soil sample used as inoculant.

In initial pH, growth of peas varied considerably between soils. This was concluded to reflect the variable efficiency of *Rhizobium* nodulating peas. When limed up to 6.5, only slight differences in plant growth were found. Also even in acidic soils there are some efficient *Rhizobium* strais present, which are favoured by liming and can contribute to high yield in optimal conditions. Further characterization of the indigenous population will show, whether liming and inoculation can be alternative measures to ensure optimal bacterial symbiont to pea in the soils studied.

THE NITROGEN-FIXING PRODUCTIVITY OF RED CLOVER VARIETIES

LEPKOVICH, I., SPIRIDONOV, A.
Grassland and Grassmaking Chair St.-Petersburg State Agricultural University 189620, St.-Petersburg - Pushkin, Russia

The experiments with red clover (*Trifolium pratense* L.) were carried out in 1987-90 in Leningrad region. The soil was dernovo-podsolic with high fertility level. The following varieties were used in stands (pure clover or in mixture with timothy): Jogeva 433, Jogeva 205 (Estonia), Hermes (Sweeden). Marino, Matri (Germany), Sivoritsky 416 - standard (Russia). The shoots were cut two or three times during growing season in 1988 and 1989. After ploughing the barley was sown in 1990. The nitrogen fixing capacity of red clover had been determined in field conditions by means of comparison the plots with and without clover plants.

The maximal level of root productivity was marked in early matured variety Jogeva 433 stand - 12.2 t per 1 ha for 3 years. The fixed nitrogen quantities were 119-363 kg per 1 ha for 3 years when stands were cut two times, and 158-547 kg - when stands were cut three times. The maximal levels of nitrogen fixation had polyploid variety Matri - 70-100 kg N per 1 ha more than diploid.varieties.

The red clover is the effective soil-improver: clover plants had provided the soil with organic material and biological nitrogen more when three cuts were made and early matured varieties were used. It was estimated that owing to biological nitrogen it is possible to obtain 32-70% of the barley (next culture - 1990) grain yield. The barley yield was 4-20% more when it was sown on the pots with clover and 3 cuts regime (in 1988-89).

DIVERSITY OF BACTERIA ISOLATED FROM ROOT NODULES OF *Onobrychis* spp. PLANTS

LIMESCHENKO, E., VOROBYOV, N., AND NOVIKOVA, N.
Research Institute for Agricultural Microbiology, St.Petersburg - Pushkin, 189620 Russia

Thirty seven strains isolated from naturally grown *Onobrychis* spp. plants, were tested on 120 phenotypic properties (growth characteristics, utilization of carbon and nitrogen sources, intrinsic antibiotic resistance), phage sensitivity and plant host range. Three groups of the strains were revealed.

The first group comprised 10 fast-growing strains (full colony development occurred after 3 days of incubation at 28°C). These strains were sensitive to *R.leguminosarum* phages, clustered together with *R. leguminosarum* strains and induced nodules on *Trifolium pratense*, *Vicia villosa* and *Phaseolus vulgaris* roots while no nodules were observed on *Onobrychis* roots inoculated with clover, vetch or bean *Rhizobium* strains.

The second group included 12 strains nodulating *Macroptilium atropurpureum*, *Lotus corniculatus*, *Coronilla varia* and *Glycyrrhiza glabra* legume plants. The strains of this group made up two clusters - together with *R. loti* strains or with *Rhizobium* strains from legumes of the temperate zone. Part of the second group strains were sensitive to *R. loti* bacteriophages. Some of rhizobia strains from the temperate zone legumes (i.e. *Astragalus*, *Hedysarum*) were able to nodulate effectively sainfoin as well.

The third group comprised 15 slow-growing strains (colony development took about 8 days). These strains were clustered separately from other *Rhizobium* or *Bradyrhizobium* strains but were able to form nitrogen fixing nodules on *Vicia villosa* roots.

The results of pot tests indicated that strains of two last groups induced more effective symbiosis with *Onobrychis* plants than the strains from the first group.

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THE INVESTIGATION AND ANALYSIS OF SOYBEAN RHIZOBIA RESOURCES IN SAN-JIANG PLAYN

LU L.-G., LU X.-C., ZHONG E-R., GAO H., LIN L.
Heilongjiag Academy of Agricultural and Reclamation Sciences,
Jiamusi, 154007 P.R.China

We report the result of morphological and biochemical identification, and serological analysis of 73 indigenous soybean rhizobia which were isolated from main soils and soybean cultivars in San-Jiang Plain, where is the gene center with very rich germplasm of soybean, the north-eastern of China. The results showed that 28,8% of the isolated strains prodused acid and could grow well in 0,45 mol/L NaCl YEM cultivation, the strains both acid-producing and salt-resisting (0,45 mol/L NaCl) would be 90,5%. Most of the isolated strains showed higher intrinsic antibiotic resistance to ampicillin and teteracycline. By cross-agglutination reaction, 32,4% of isolated strains were divided into 7 serotypes. The predominant serotype are 2048 and 10324, which represented 19,1% and 5,9% of the strains examinated. Research results show that there is a lot of types with a widespread distribution and display the diversity of germplasm for indigenous rhizobia. The resources' characteristics and ecological distribution should be related with soybean variety and soil-ecotopy.

ANNUAL MEDICS HAVE SPECIFIC SYMBIOTIC REQUIREMENTS.

MATERON, L.A., ZAKLOUTA, M.
International Center for Agricultural Research in the Dry Areas
(ICARDA) P.O. Box 5466, Aleppo, Syria

Experiments conducted with 247 isolates of *Rhizobium meliloti* from soil samples collected from diverse arable areas in west Asia (Cyprus, Jordan, Lebanon, Syria and Turkey) were assessed to define their symbiotic characteristics and degree of host specificity on six species of annual *Medicago* (*M. rigidula* (L.) All., *M. noeana* (Boiss.), *M. rotata* (Boiss.), *M. orbicularis* (L.) Bart. and Australian cultivars of *M. truncatula* Gaertn. cv. Jemalong and *M. polymorpha* L. cv Circle Valley). Host species responses were evaluated based on nodule appearance and plant biomass. The test soils contained populations of *R. meliloti* with different ability to nodulate these specific legumes and fix N_2. The isolates were classified into four categories: highly effective, partially effective, ineffective and non-nodulating. The number of *R. meliloti* in the soil was $<7.2 \times 10^5$ g^{-1}. Soils from Syria, Jordan and Turkey contained higher proportions of ineffective rhizobia than those from Cyprus and Lebanon. Despite the widespread presence of ineffective rhizobia, highly-effective strains were identified from all soils and all countries for each of the six species of *Medicago*. The proportion of highly-effective rhizobia nodulating all hosts was <10% of the total rhizobial population in the samples from each country. A significantly higher proportion of partially-effective rhizobia was present in most samples for each host.

SELECTION OF GOAT'S RUE (*GALEGA ORIENTALIS* LAM.) FORINCREASING EFFICIENCY OF SYMBIOTIC NITROGEN FIXATION.

METLITSKAYA, J.
Jõgeva Plant Breeding Institute EE2350,Jõgeva, Estonia.

An important property of goat's rue (*Galega orientalis* Lam.) is the ability to form symbiosis with nodule bacteria *Rhizobium galegae*.The aims of this study were to evaluate the variability of the variety Gale by nitrogen fixing capacity and to find out possibilities of increasing the symbiotic efficiency of goat's rue by selection. The nitrogen-fixing activity was determined by the acetylene reduction assay (Hardy et al., 1968).

The intraspecific variability by the nitrogen-fixing capacity in sterile experiment conditions was high (coefficient of variation 77,7...127,3%), approximately 15% of plants were entirely incapable of fixing nitrogen (Fix$^-$). Out of 1145 plants analyzed, 109 Fix$^-$ plants and 117 plants with maximal nitrogen-fixing activity (Fix^{++}) were picked out. The plants of those two groups were first planted into light soil and thereafter replanted into field conditions. At the end of the first year of growth, only 34% of the Fix$^-$ plants and 51% of the Fix^{++} plants had a good development (3...8 stems with the height of 16...25 cm). In the second year of growth the Fix^{++} plants were already clearly superior to those Fix$^-$ in the yielding ability and velocity of entering the generative stage. As an initial material for the further selection of goat's rue, a number of Fix^{++} plants with good basic selection parameters were picked out.

The selection of goat's rue (*Galega orientalis* Lam.) for high nitrogen fixing activity in the conditions of microvegetative experiment could be used in the selection of goat's rue for a higher symbiotic efficiency.

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SOYBEAN BRADYRHIZOBIA POPULATION DYNAMICS: THE PRESENCE OF HIGHLY REITERATED RS-ALPHA-POSSESSING FIELD ISOLATES IN JAPAN

MINAMISAWA, K.[1,2], NAKATSUKA, Y.[1], SEKI, T.[1], ISAWA, T.[1]
[1]School of Agriculture, Ibaraki University, Ami, Ibaraki, 300-03 Japan.
[2]Institute of Genetic Ecology, Tohoku University, Sendai, Miyagi, 980 Japan.

We characterized 213 isolates of soybean bradyrhizobia indigenous to 6 field sites (Tokachi, Nakazawa, Nagakura, Ami, Fukuyama and Ishigaki) in Japan by using two *B. japonicum* hybridization probes, RS-alpha and RS-beta (Kaluza, K. et al., 1985). Diversity and dominance in RSs (RS-alpha and RS-beta)-specific hybridization patterns (Minamisawa et al.,1992) were observed, which depended on individual field. In particular, 21 isolates from Tokachi, Nakazawa and Nagakura fields showed uncountable multiple bands of RS-specific hybridization, which can be easily distinguished from normal hybridization patterns. These isolates are designated HRS (Highly Reiterated RS-possessing) isolates in this study.

The intensity of hybridization signals was compared with that of USDA110, which possesses 12 copies of RS-alpha and 6 copies of RS-beta. The estimated copy numbers of RS-alpha and RS-beta in HRS isolates were much higher than those of the normal isolates. Among HRS isolates, Niigata-type HRS isolates from Nakazawa and Nagakura fields possessed significantly higher number of RS-alpha copies (86-175 copies per genome).

We obtained the physical evidence for genome rearrangements in HRS isolates. (1) A part of Niigata type-HRS isolates showed shift and duplex of *nif* and *hup*-specific hybridization bands. (2) Analysis of DNA duplex by denaturation and renaturation of total DNA and by treatment with S_1 nuclease shows that inverted repeat structures of RS-alpha were present on the genome of Niigata-type HRS isolates. In addition, inverted repeats of RS-beta and unknown sequence (2.0 kb) were detected on the genome of all HRS isolates tested. (3)We also found a tandem repeat RS-alpha dimer [(RS-alpha)$_2$] on the genome of HRS isolates. The structure of (RS-alpha)$_2$ is similar to that of (IS*30*)$_2$, which gives rise to a burst of transpositional rearrangements in *E. coli* (Orasz et al., 1993).

Preliminary surveys of symbiotic phenotypes showed no difference between HRS and normal isolates with respect to nodulation, nitrogen fixation and hydrogen uptake. On the other hand, we found that HRS and normal isolates differ in free-living growth. HRS isolates exhibited slower growth and/or longer lag time than the normal isolates.

These results suggest that RSs-mediated genome rearrangements occur in HRS isolates, which may decrease their growth rate in culture and generate genetic diversity within the populations of soybean bradyrhizobia.

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PARTITIONING OF ^{15}N LABELLED MINERAL NITROGEN AND N UPTAKE BY *ACACIA*, *EUCALYPTUS* AND *ATRIPLEX*

MOHAMMED, I., SALOUA, B., AND SALEMA, M.P.
Faculte des Sciences, PB 4010 BeniMhamed, Meknes, Morocco; Soil Fertility and Irrigation Section, I.A.E.A., Wagramerstrassse 5, POB 100, A-1400 Vienna, Austria

In many regions of Morocco, the soil fertility have eteriorated in recent years owing to loss of productivity and erbaceous cover. Restoration of fertility and productivity may e best achieved by establishment of Acacia and Atriplex species. n experiment was conducted in an arid site on a sandy clay loam oil under 178 mm annual rainfall. Five trees of Atriplex ummularia, Acacia cyanophylla, A. cyclops and Eucalyptus orquata received 4 g of ammonium sulfate enriched with 10 at. ^{15}N excess diluted in water. The age of the trees was five ears. Tweleve months after, each tree was destroyed and divided nto five parts from up to the roots. Each part was divided into eaves and stems. Dry weight, total N and % ^{15}N were determined.

The establishment and growth of Atriplex were rapid showing hat this plant is very adapted to arid environments. Trees ccumulated enough ^{15}N excess in all parts which had different oncentrations of ^{15}N. All parts of Atriplex were less oncentrated in ^{15}N than Eucalyptus and Acacia showing that triplex have high ability to absorb and assimilate soil nitrogen nd also fix atmospheric N_2. Acacia cyclops fixed only 11% of its . Acacia cyannophylla fixed 41% of its N. But, A. cyclops took p more N from soil than A. cyanophylla. Atriplex was also fixing l$_2$ because its ^{15}N concentration was very low. Atriplex contained ess than half of their dry matter in woody tissue and was more oncentrated in N than A. cyanophylla.

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USE OF PCR-RFLP ANALYSIS TO DISTINGUISH AMONG *FRANKIA* STRAINS IN NODULES OF RED ALDER

MYROLD, D.D. (1), MARTIN, K.J. (2), RITCHIE, N.J. (1)
(1) Department of Crop and Soil Science, Oregon State University, ALS 3017, Corvallis, OR 97331-7306, U.S.A.
(2) Mantech Environmental Services, Inc., Corvallis, OR 97333, U.S.A.

The lack of easy, specific isolation and detection methods has hampered studies of *Frankia* strains in natural plant-soil systems. In particular, it would be useful to distinguish among closely related strains within the same host-specificity group. For example, this would allow studies of strain competition to be done. The objective of our study was to develop a DNA-based method to discriminate among *Alnus*-infective *Frankia* strains and apply this in a field inoculation study. We developed a method to differentiate among some *Frankia* strains that nodulate *Alnus* spp. This method works with DNA extracted from pure cultures of *Frankia* strains and with DNA isolated from lobe tips of root nodules. We used the polymerase chain reaction (PCR) to specifically amplify a 948-bp portion of the *Frankia* glutamine synthetase (*gln*II) gene. The resulting PCR product was then cut with one of several restriction enzymes and the resulting DNA fragments were separated by agarose gel electrophoresis to give an RFLP (restriction fragment length polymorphism) pattern. *Hae*III and *Hha*I had the greatest discriminating power of the restriction enzymes used. The PCR-RFLP patterns were different among host-specificity groups and also allowed us to discriminate among some *Alnus*-infective *Frankia* strains. We applied this PCR-RFLP method to distinguish among *Frankia* strains in nodules of red alder (*Alnus rubra* Bong.) seedlings. An inoculation trial was set up in a fumigated nursery soil (Martin et al., 1992). Plots were inoculated with either *Frankia* strain ArI5, strain AvcI1, equal amounts of both strains (dual inoculum), or left noninoculated (control). After one growing season, we harvested the red alder seedlings, extracted DNA from their root nodules, and used the PCR-RFLP method to identify the *Frankia* strains inhabiting the root nodules. In general, we found the expected PCR-RFLP patterns in plots inoculated with only one strain of *Frankia*, although there was a background of native *Frankia* that had the ArI5 pattern. The ArI5 pattern dominated in the dual inoculum plot, suggesting that strain ArI5 was more competitive for nodulation. We also observed some unique PCR-RFLP patterns in the control plots, suggesting that there were some unique native strains of *Alnus*-infective *Frankia*. This successful application of PCR-RFLP methods using the *gln*II gene, or adapting it for use with other *Frankia* genes, demonstrates the feasibility of studies about the diversity of *Frankia* in nature without having to first isolate the microbiont.

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IMPROVEMENT OF NITROGEN FIXATION IN PEAS (*Pisum sativum* L.)

NAUMKINA T.S.
All Russia Research Institute of Legumes and Groat
Crops, Orel, p/b Streletskoye, 303112, Russia

Essential improvement of nitrogen fixing ability in peas can be achieved by creation of high copmlementary pairs of macro- and microsymbiont. In All Russia Research Institute of Legumes and Groat Crops we worked out and fulfilled breeding programme of creation of pea varieties, resistant to domestic populations and succeptable to introduced rhisobia strains. The experiment was based on Prof. N.M.Chekalin (1989) methodics: introgression of *sym2* alleles into genotype of the best commercial pea varieties for obtaining the isogenic lines of these varieties. Isogenic lines must be resistant to rhisobia strains and form active symbiosis with A-1 strain. Afghanistan lines k-1887, k-6559, l-2150 (All Russia Research Institute for Plant Breeding) are donors of *sym2* allele. After 6-times backcrosses and series of selections, without nitrogen background, we isolated 2 plants, phenotypically similar to paretal Nord variety, but with genotype *sym2sym2*. Plants of new line are absolutely resistant to European rhizobia strains. Comparative studies of Nord variety and new line in the presence of mineral nitrogen and without it, showed that seeds of this line should be treated with strains of *Rhizobium leguminosarum* bv. *viciae* A-1, A-III. Strains of these rhizobia form active symbiosis with plants and afford to form seed productivity 3,6 g per plant. This is 57% more than by Nord variety, and without mineral nitrogen in soil.

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TAXONOMY OF RHIZOBIA ISOLATED FROM THE ROOT NODULES OF LEGUMINOUS TREES IN THE SUDAN

NICK, G.[1], JARVIS, B.D.W.[2], TIGHE, S.W.[3], NIEMI, M.[4],
DE LAJUDIE, P.[5] AND LINDSTROM, K.[1]
(1) Department of Applied Chemistry and Microbiology, POB 27, Fin-00014 University of Helsinki, Finland, (2) Department of Microbiology and Genetics, Massey University, Palmerston North, New Zealand, (3) Analytical Services Inc., POB 626, Essex Junction, VT 05453, USA, (4) National Boards of Waters and the Environment, POB 250, FIN-00101 Helsinki, Finland, (5) Laboratoire de Microbiologie des Sols, ORSTOM BP 1386, Dakar, Senegal, West Africa

Molecular methods and fatty acid analysis were used to further characterize rhizobia isolated from the root nodules of *Acacia senegal* and *Prosopis chilensis*. In this work the relationships between the tree rhizobia, rhizobia isolated from different plants and reference strains representing recognized rhizobial species were analyzed by total cellular fatty acid analysis, DNA-DNA dot-blot hybridizations, restriction fragment length polymorphism analysis of amplified 16 S rDNA obtained by PCR, and by REP and ERIC PCR fingerprints. Also the methods used in this study showed that the Sudanese tree rhizobia are diverse, even though the majority of the strains seemed to belong to two main clusters. The strains of these main clusters belong to the same phylogenetic branch as *Sinorhizobium meliloti* and *S. fredii* and might represent two new species. Some of the strains seem to belong to the recently described two new species *S. teranga* and *S. saheli*. One strain may be a *S. meliloti* B type and two strains belong to the Senegalese group U. Part of the strains resemble agrobacteria (fig.1). The taxonomic position of some of the strains still remains unclear.

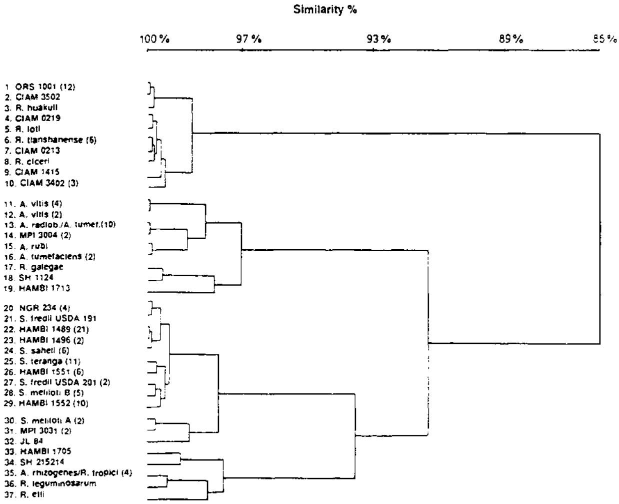

Fig.1. UPGMA dendrogram based on genetic distances derived from PCR-RFLP analysis of the amplified 16 S rDNA. The number of strains which gave the same profiles is indicated in parentheses.

TAXONOMY OF *Rhizobium* STRAINS FROM LEGUMES OF THE TEMPERATE ZONE

NOVIKOVA, N. (1), LIMESCHENKO, E. (1), BUSTOS, P. (2),
RODRIGUEZ, O. (2), DAVILA, G. (2), AND VOROBYOV, N. (1)
(1) Research Institute for Agricultural Microbiology,
St.Petersburg - Pushkin, 189620 Russia
(2) Centro de Investigacion sobre Fijacion de Nitrogeno, UNAM,
Cuernavaca, Morelos, Mexico.

Phenotypic properties of 53 *Rhizobium* strains isolated from root nodules of the temperate zone legumes *Astragalus* spp., *Oxytropis campanulata*, *Hedysarum alpinum*, *Ononis arvensis*, *Glycyrrhiza* spp., and *Coronilla varia*, were compared with those of other *Rhizobium*, *Bradyrhizobium* and *Agrobacterium* strains by means of numerical taxonomy. The dendrogram showed that the new strains formed two clusters separated from both *Rhizobium* and *Bradyrhizobium* spp. Group 1 included rhizobial strains originated from different geographical regions with a temperate climate, while group 2 included strains from the same geographical origin, South Siberia. The diversity of these groups was confirmed with DNA:DNA hybridization experiments with reference DNAs from *R. loti* NZP 2213^T and *R.huakuii* CCBAU 2609^T. The sensitivity of these strains to bacteriophages of *Rhizobium loti*, *R.meliloti*, *R.galegae* and *R.leguminosarum* was studied. Phages specific to *R loti* strains were shown to induce the phage lysis on several *Astragalus*, *Hedysarum* and *Ononis* rhizobia as well as on *R.huakuii* strains. Ten *R.loti* strains tested for nodulation abilities on the plant host under investigation were able to develop nitrogen-fixing nodules on the *Ononis arvensis* roots. Bacterial isolates from *Astragalus* and *Hedysarum* nodules were observed to cross-nodulate their plant hosts as well as *Oxytropis campestris*, *Glycyrrhiza uralensis* and *Ononis arvensis* plants. The symbiotic genes of these strains seem to be located on the plasmids. The phylogenetic analysis performed by partial sequencing of a 260 bp 16S rRNA fragment for the strain CIAM 3502 (*Ononis arvensis*) demonstrated its inclusion in the *R.loti-R.huakuii-R.ciceri* lineage and its diversity from other recognized rhizobia species.

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EFFECT OF SODIUM CHLORIDE ON *GLYCYRRHIZA URALENSIS-RHIZOBIUM* SYMBIOSIS

NOVIKOVA T.I., GORDIENKO N.Y., TSEITLINA V.R.
Central Siberian Botanical Garden, Zolotodolinskaya 101, Novosibirsk, 630090, Russia

Nodule initiation in the legume-*Rhizobium* symbiosis involves a complex interaction among the host plant, rhizobial partner and enviroment. It is well known that salt stress inhibits nitrogen fixation by legumes. *Glycyrrhiza uralensis Fish.* which usually grows in saline areas may be adapted to fix N_2 successfully under these conditions. Our research was focused on exploring the salt effects on early stages of licorice-*Rhizobium* interaction, formation of nodules, their nitrogen fixing activity and structure. The low salt concentration (34 mM NaCl) stimulated the nodulation and N_2 fixation. Increasing chloride salanization (102–204 mM) reduced nodulation by inhibiting early simbiotic stages such as curling of root hairs and formation of infection threads. The nodules formed under 170–204 mM NaCl were examined using light, electron microscopy and computer morphometric analysis. These nodules had relatively smaller infection area in the central zone, lower density of bacteroids per infected cell and smaller size of bacteria, than those formed under non-saline conditions. As for peribacteroid space, it was 2.4 times greater than that under control. Nodulation of licorice was completely inhibited when salinity was raised to 238 mM. This result showed that *Glycyrrhiza uralensis-Rhizobium* symbiosis was a rather salt-tolerant system. The nodules formed under non-saline conditions were more sensitive to NaCl-stress than those developed in the presence of salt. After 170 mM NaCl-treatment, 60 % inhibition of nitrogenase activity was registrated 96 h later. This reduction of nitrogen fixation was due, in part, to plasmolysis in infected cells, degradation of peribacteroid membranes, and structural damage of bacteroids.

ASSESSMENT OF NITROGEN FIXATION POTENTIAL OF KENYAN *LEUCAENA LEUCOCEPHALA* CULTIVARS IN AN ALFISOL

ODEE D.W.
Biotechnology Division, Kenya Forestry Research Institute, P O Box 20412,
Nairobi, KENYA.

Five Kenyan *Leucaena leucocephala* (Lam.) De Wit cultivars viz:- Siakago, Isiolo, Baobab, Gede and Muka Mukuu were screened for effectiveness with selected *Rhizobium* sp. strains Ll1, Ll2, Ll3, Ll4 and TAL 1145. The experiment was carried out in potted Muguga soil (Alfisol) supplemented with a basal fertiliser and grown for 12 weeks under glasshouse conditions.

Results showed that there was a significant strain effect ($P = 0.05$) on shoot, root and nodule biomass and N content. TAL 1145 was the most effective and competitive strain against the soil borne rhizobia in terms of accumulated N (2.1 and 1.8 shoot and root %N respectively; 163 mg plant^{-1} total N). Shoot biomass was greatest with strain Ll3 (5.4 g plant^{-1}) and lowest with soil borne rhizobia i.e., uninoculated control (4.5 g plant^{-1}).

Cultivar effect was significant only with shoot biomass and height at harvest. Isiolo provenance accumulated the greatest biomass (5.3 g plant^{-1}) and attained highest growth (34.6 cm). Cultivar and strain interaction was also significant only with shoot biomass.

The lack of significant variations between the Kenyan *L. leucocephala* cultivars in growth and nitrogen fixation attributes suggested that they may have some common descent.

GENOTYPIC DIVERSITY OF A *RHIZOBIUM MELILOTI* POPULATION RECOVERED FROM DIFFERENT *MEDICAGO SATIVA* CULTIVARS IN NORTHERN ITALIAN SOIL

D. PAFFETTI (1), C. SCOTTI (2), P. ROTILI (2), S. FANCELLI (1) AND M. BAZZICALUPO (1)
(1) Dipartimento di Biologia Animale e Genetica, Via Romana 17, I-50125 Firenze Italy. E-mail: MARCOBAZZI@DBAG.UNIFI.IT
(2) Istituto Colture Foraggere, Viale Piacenza 29, I-20075 Lodi Italy

The population genetics of the *Rhizobium* spp. has been extensively studied and a high degree of genetic diversity and absence of linkage disequilibrium was found. It is not known, however, whether the genetic polymorphism within a natural *Rhizobium* population corresponds to preferences for nodulating particular genotypes of the compatible host. Moreover, it is not known what is the influence of the soil and of the plant genotype on the evolution of the population structure.

A major advancement in population genetics of *Rhizobium* is expected from the introduction of molecular methods based on the analysis of DNA molecules.

In order to investigate the relationship between bacterial and plant genotypes, the genetic structure a natural population of *R. meliloti* was determined after recovering the bacteria from the nodules of three cultivars of *Medicago sativa* with distinct geographic origin and planted in two different Northern Italian sites.

92 phenotypically indistinguishable strains, isolated from nodules, were analysed for DNA polymorphism with three methods: Random Amplified Polymorphic DNA (RAPD) of total genomic DNA, Restriction Fragment Length Polymorphism (RFLP) of a 25 kb region of the *pSym* plasmid containing *nod* genes and RFLP of the intergenic region between 16S-23S rDNA (ARDRA). Data obtained and the results of their analysis thereupon, suggest the following conclusions:

1) A great level of genetic diversity was found among the 92 strains, in particular as revelead by RAPDs.
2) The genetic diversity resides on all genome (RAPD), but seems distributed either on the chromosome (intergenic region between 16S-23S rDNA) and even more on the plasmid (25 kb region of the *pSym* plasmid containing *nod* genes)
3) This genetic diversity is not equally distributed in the population:
   a) The genetic diversity is lower in the sub-population for each soil than in total population. Moreover the sub-population of soil A is genetically different from that of soil C.
   b) The different plant genotypes (varieties) seem to be nodulated preferentially by particular bacterial genotypes.

GROWTH INTERACTION BETWEEN *NOSTOC* AND *RHIZOBIUM*

PANKRATOVA, YE.M. KALININ, A.A., KOVINA. A.L.
Agricultural Academy at Kirov, 610017 Russia

In nature, cyanobacteria exist in associations with different saprotrophic bacteria, which could enter into cells slime of these organisms. Under laboratory conditions the associative N_2 -fixing system can be established by *Nostoc paludosum* st. 18 and *Rhizobium galegae* st 0702/ *R. leguminosarum* st. 1022, which was introduced into of the cells slime of cyanobacteria in place of natural satellites. The growth of the association *Cyanobacteria-Rhizobium* was observed in batch culture in the nitrogen free Gromov N6 medium (1956) and on the sterile bog moss (*Sphagnum*), pH up 6.8 and wetting by the medium of Gromov. The experiment show that cyanobacteria do not eliminate the cells of *Rhizobium* : the amount of its cells increases simultaneously with the increase the cells of the other partner. *N.paludosum* can exudate a portion of carbonaceous compounds during its growth. For example, the amount of nitrogen in exudation was about 20-30% of the total amount of nitrogen fixed by *Nostoc.* These compounds can be absorbed by *Rhizobium*. In culture we can watch homeostasis of association when *N.paludosum* reaches exponential growth. If the mass of cyanobacteria is too small, Rhizobium growth is limited by the presence of organic matter in the medium. Association between *Nostoc* and *Rhizobium* has of higher nitrogenase activity and rate protein syntesis than cyanobacterial monoculture. When aseptic culture *N.paludosum* grown in liquid medium alone its ARA was 0.42 nmol C_2H_4 /mg protein/min (age 15 day); in associative system ARA could reach 1.09 nmol C_2H_4 /mg protein/min. Rapid growth was observed on the bog moss. Association, which was formed on the surface of moss, survived after six month of desiccation and recovered when water was re-supplied. Also this association was remained in soil longer, than individual *Rhizobium*. The nitrogen-fixing association has been investigated with the purpose of a potential utilization it as a biological preparation for leguminous crops. Work was undertaken in pot and field on *Galega orientalis* and *Pisum sativum* to study the role of biopreparation. The treatments consisted of inoculation with *Rhizobium* alone and association of *Rhizobium* and *Nostoc*. For example, the effect of *N.paludosum* on Galega orientalis was 1.7 ton/ga hay and 5.8 kg/ga seed under field conditions over treated *Rhizobium* alone.

BIOLOGICAL NITROGEN IN AGRICULTURE OF THE UKRAINE

PATYKA, V.F., SHERSTOBOEVA, E.V., TARARICO, A.G.
Ukrainian Academy of Agriculture Sciences, Kiev, 252010, Ukraine

We demonstrated that the rhizobia application in the Ukraine increases crop yield of soybean by 3-6 c/ha, peas - by 1,5-2 c/ha, green mass of bean grass - by 80-100 c/ha, alfalfa and clover hay - by 6-8 c/ha. In this case protein content in the grain increases by 2-3%. It was shown, that the usage of biopreparations of associative microorganisms (rhizoagrin, rhizoenterin, flavobacterin and azorhizin) prepared on the basis of our own strains, for crop cultures gives yield increment by 8-20% and in individual cases increase was 10-12 c/ha in the South of Ukraine.

We hope that increases of input on N_2-fixation into the nitrogen balance may be improved if we shall rise compatibility of partners in symbioses and associations. We developed soybean cultivars (Arcadia Odescaya, Odescaya 124, Krepysh and others) with abnormally high intensity of symbiotic nitrogen fixation.

THE TRANSFORMATION OF HERBICIDES BY NODULE BACTERIA AND LEGUME - *RHIZOBIUM* SYMBIOTIC SYSTEM.

PAROMENSKAYA, L.N., KRUGLOV, Yu.V.
Research Institute for Agricultural Microbiology, Podbelsky Sh. 3, St.Petersburg-Pushkin 189620, Russia.

The ability of nodule bacteria and symbiotic legume - rhizobial systems to degrade herbicides was studied. In the pot experiments with the use of herbicide 2,4-D labeled with ^{14}C in the ring it was established that after the leaves were treated with the herbicide it moved into the root system accumulating in the root nodules where it was subjected to active transformation. The radiometric analysis of the extracts taken from the nodules showed that a week after the treatment over 90% of ^{14}C which entered the nodules is in the form of the herbicide metabolites. Similar data were obtained on the herbicide treflan.The content of this herbicide in the root system of soya inoculated with nodule bacteria turned out to be considerably higher as compared with noninoculated plants. Transformation of herbicide takes place only in the roots of symbiotrophic soya which was confirmed by the presence of metabolyte 2-nitro- 4-triphtormethyl 6-amino-n-propylaniline. A much more active destruction of herbicide occurred in the root nodules in which 3 metabolites were detected. The results obtained in the model experiments with nodules detached from the roots of leguminous plants confirmed the active part which the nodules take in degradation of 2,4-D, prometryne and treflan. In the laboratory experiments on liquid nutrient media the ability of the nodule bacteria to degrade the herbicides 2,4-D and prometryne were studied. The residues of herbicides were determined using the technique of gas-liquid and thin layer chromatography. It was found that certain strains of nodule bacteria degrade these herbicides. Degradation of herbicides proceeds according to a cometabolism type in presence of additional energy material. The character and effectiveness of the process depend on the bacteria type and strain. In the laboratory experiments a conjugative transfer of plasmid *RP4:TOL* from *Pseudomonas putida* into the cells of *Rhizobium trifolii* was done. The transconjugants obtained acquired an ability to oxidize toluene and 3-chlorbensene to catechole in the presence of co-substrata. As this took place the resistance of legume - rhizobial symbiotic system to herbicide 2,4-D increased after inoculation of clover with transconiugants. The use of the strains degrading herbicides for inoculating the legumes enhances the resistance of the symbiotic nitrogen fixing system to herbicides and accelerates the detoxication processes in soil, which is of significant value for obtaining clean product and for environment protection.

ULTRASTRUCTURAL CHANGES IN PEA NODULES AND *RHIZOBIUM* CELLS INDUCED BY HERBICIDES

PAROMENSKAYA, L.N., LEBSKY, V.K., KOZLOVA, E.D.
Research Institute for Agricultural Microbiology, Podbelsky Shosse 3, St.Petersburg - Pushkin 8, 189620, Russia.

Herbicides are known to be physiologically - active compounds, which exert a multilateral influence on those aspects of both macro- and microsymbiont metabolism, that are directly or indirectly connected with the process of symbiotic nitrogen fixation (photosyntesis, process of phosphorilation, oxidative metabolism, ets.). So, it can be suggested that herbicides are a potential danger for legume - *Rhizobium* symbiosis.

An influence of herbicides MCPB and prometryne on the symbiotic nitrogen fixation and ultrastructure of nodules was studied. It was found that negative action of herbicides on symbiotic nitrogen fixation (decrease of nitrogenase activity and "biological" nitrogen contents in plants) is accompanied by significant changes in the ultrastructure of nodules.

Nodules from pea plants, treated with herbicides, were characterized by the reduced volume of nitrogen - fixing zone and by the reduced number of infected cells. Uninfected plant cells in nodules contained numerous large starch granules. Infected plant cells exhibited both normal bacteroids and involution forms. Destruction of plant cytoplasm and nucleus as well as lysis of bacteroids induced by herbicides were similar to those observed in the course of nodule senescense.

In laboratory experiments the effects of herbicides prometryne and MCPB on biochemical activity and ultrastructure *Rhizobium leguminosarum* strains have been studied. The character of change of dehydrogenase and catalase activities was determinated by chemical structure of herbicide, its concentration and also strains of nodule bacteria. Free - living cells of *Rhizobium leguminosarum* cultivated in presence of intoxic concentration of herbicides contained spherical structures similar to ribosomes but distinct from them by bigger size. In addition, the cells were characterized by the elevated amount of lipids. Toxic concentration of herbicides caused an increase of the surface and changed the shape of bacterial cells. Treated cells, in contrast to untreated cells, contained myeloid - like structures inside. In addition, we found that the herbicides caused the budding of a some bacterial cells.

COMPUTER SIMULATION MODEL OF BIOLOGICAL NITROGEN FIXATION BY FORAGE LEGUMES (ON EXAMPLE OF ALFALFA).

POLUEKTOV R.A. (1), KHVOROVA L.A. (2).
(1) Agrophysical Research Institute, Grazhdansky prosp. 14, St.Petersburg 195220, Russia.
(2) Altaj State University, Dimirova 66, Barnaul, 656099, Russia

The model elaborated describes seasonal dynamics of symbiotic nitrogen fixation by legumes. It consists of two units: the unit of phase nodules structure and the unit calculating the rate of a fixed nitrogen entering into a plant. The processes of formation of nodules centers on the growing roots, their transfer in the nitrogen fixation stage, and nodules dying off are simulated in the first unit. The second unit includes calculation of nodules mass according to stages of development and the intensity of symbiotically fixed nitrogen in the plant. Dynamics of the nodule phase structure on the each phase of nodule tissue for a model time step is described by balance relations. The rate of the formation of new nodules is assumed to be proportional to increase in root biomass. The main factor limiting the process of nitrogen fixation, and consequently the rate of nodule transfer in a nitrogen fixation stage is carbohydrate input to the nodule formation sites. So it is assumed that the rate of the nodule transfer in the fixation stage is caused by availability of photo-assimilates. The rate of nitrogen reduction to ammonium is determined by the current biomass of the nodules.

The units elaborated are included as the submodels into the total (summary) model describing plant growth and development as well as yield formation of alfalfa under the field conditions. The model as a whole consists of the units describing following processes: radiation regime of plant canopy, water and heat dynamics in soil, plants and the atmosphere, soil nitrogen transformation, photosynthesis and photo respiration, plant development, and dark metabolism taking into account N/C interaction in the plants. Model is written in Turbo-Pascal code and implemented on PC/AT 286 computer.

USE OF WILD-GROWING ALFALFA, SWEET CLOVER AND VETCH SPECIES AS THE TRAP-HOSTS FOR *RHIZOBIUM* STRAINS WHICH FORM THE EFFECTIVE SYMBIOSES WITH CULTURED SPECIES: *MEDICAGO VARIA* MART. AND *VICIA VILLOSA* ROTH.

PROVOROV N.A.[1], BERIOZKO A.M.[2], KURCHAK O.N.[1], SIMAROV B.V.[1]
[1] All-Russia Research Institute for Agricultural Microbiology, Sh. Podbelsky 3, St.Petersburg - Pushkin 8, 189620, Russia;
[2] Mountain Botanical Garden, Gadzhieva 45, Mahachkala, Daghestan, 367025, Russia

From the nodules of 12 wild-growing alfalfa (*Medicago* L.) and sweet clover (*Melilotus* Mill.) species collected in Daghestan (the Caucasian centre for taxonomic diversity of *Medicago* spp.), 112 *Rhizobium meliloti* strains were isolated. Among them, 37 strains were as effective as the type strain CIAM1723, while 5 strains (1 - *Medicago daghestanica* Rupr. ex. Boiss., 1 - *M. hemicaerulea* Sinsk., 1 - *M. denticulata* Willd., 2 - *Melilotus officinalis* (L.) Pall.) exceeded this strain in efficiency of symbiosis with the cultured alfalfa *M. varia* Mart. (in aseptic trials). Among 16 *R. leguminosarum* bv. *viceae* strains isolated from the nodules of 8 *Vicia* L. species collected in Mountain Tadjikistan, 11 strains were as effective as the type strain CIAM0610 while 1 strain (*V. peregrina* L. isolate) exceeded this strain in efficiency of symbiosis with the hairy vetch, *V. villosa* Roth. Symbiotic efficiency of the analyzed *R. meliloti* and *R.l.*bv.*viceae* strains did not depend on the taxonomic position of the *Medicago* and *Vicia* species from which the bacteria were isolated. At the same time, proportions of the strains resistant to streptomycin and chloramphenicol were significantly higher for the subgenus *Falcago* species than for the other alfalfa subgenera (*Orbicularia, Lupularia, Spirocarpos*). These data suggest that the species of subgenus *Falcago* can select preferentially some *R. meliloti* genotypes from the natural populations, but this selectivity does not concern the specificity of formation of the effective symbiosis with *M. varia*. The endemic Caucasian alfalfa species *M. daghestanica* and *M. glutinosa* Grossh. formed more effective symbioses with the "homologous" *R. meliloti* strains ("own" isolates of the appropriate species) than with the "heterologous" *R. meliloti* strains (isolated from the other plant species). At the same time, the ecologically promiscuous alfalfa *M. hemicaerulea* formed equally effective symbioses with homo- and heterologous *R. meliloti* strains. These data suggest a possibility of using the wild-growing legumes as the trap-hosts for highly-effective rhizobia strains for inoculation of the test-hosts from the same cross-inoculation groups.

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SYMBIOTIC EFFICIENCY IN THE LEGUMES CULTIVATED IN MIDDLE ASIA - CHICKPEA (*CICER ARIETINUM* L.), MUNGBEAN (*PHASEOLUS AUREUS* ROXB.) AND PEANUT (*ARACHIS HYPOGAEA* L.)

PROVOROV N.A., NOVIKOVA N.I., LIMESCHENKO E.V., KOZHEMIAKOV A.P. - All-Russia Research Institute for Agricultural Microbiology, Sh.Podbelsky,3, St.Petersburg, Pushkin, 8, 189620, Russia;
SAIMNAZAROV U.B., BAHROMOV I.U., PULATOVA D.Z., RASULOV A.S., MURODOV M.M. - Institute for Microbiology, Abdulla Kadyri 7b, Tashkent, 700128, Uzbekistan;
KURBANOV G.A., OMONTURDIEV B.O. - Institute for Plant Breeding, Tashkent area, Botanika, 702134, Uzbekistan

In 2 field trials conducted in Uzbekistan, inoculation of chickpea (*Cicer arietinum* L.) with the type *Bradyrhizobium* sp. strain CIAM2102 lead to increase (in average for 3 cultivars) of seed mass by 6.6-21.4%, of shoot mass by 10.0-13.8%. A more high symbiotic efficiency was detected in mungbean (*Phaseolus aureus* Roxb.) and peanut (*Arachis hypogaea* L.): inoculation with the type *Bradyrhizobium* spp. strains isolated from these legumes (CIAM1901 and CIAM0104, respectively) increased the seed mass by 33.9-47.8% and 52.3-56.1%, shoot mass by 42.9-63.0% and 20.0-109.1% (in average for 2 and 3 cultivars, respectively). The number of nodules was increased due to the rhizobia inoculation by 27.9-43.8% for chickpea, by 72.7-249.4% for mungbean and by 700.3-828.9% for peanut. The highest correlations between the analysed parameters of symbiotic activity were obtained for peanut ($r=+0.94...+0.99$), the lowest - for chickpea ($r=+0.35...+0.84$). In mungbean and chickpea, seed and shoot yields correlated significantly ($r=+0.66...+0.95$; $P_o<0.05$) with number of large pink nodules, while their correlations with number of small white nodules were non-significant. In peanut, seed and shoot yields correlated significantly with the numbers of both large and small nodules ($r>0.90$; $P_o<0.01$). Two-factor analysis of variance demonstrated that in peanut, both seed and shoot yields are controlled mainly by the rhizobia strain genotype (62.9-92.7% of total variation of these parameters). In mungbean the seed mass was controlled mainly by the cultivar genotypes while the shoot mass - by strain genotype (53.8% and 73.1% of total variation respectively). In chickpea the shoot mass was controlled mainly by the cultivar genotypes (89.3% of total variation), while the balance between additive and non-additive actions of partners' genotypes on the seed mass differed greatly in three independent experiments. From Uzbekistan soils we have isolated five promising fast-growing mungbean strains (in aseptic tube tests the shoot mass of the host increased after inoculation by 41.3-99.5%) and four fast-growing peanut strains (shoot mass was increased by 68.2-82.7%).

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ADDED NITROGEN INTERACTIONS IN ^{15}N EXPERIMENTS WITH *AZOLLA* IN FLOODED RICE SOIL

RABEHARISOA, R.L.
Universite d'Antananarivo, Laboratoire des radioisotopes,
B.P. 3383, 101 Antananarivo, Madagascar

The effect of different methods of use of ^{15}N in the studies of the mineralization of nitrogen in *Azolla* and in soil was studied in incubation experiments.

Azolla, as nitrogen source, was incorporated either after its labelling with ^{15}N (direct method), or without labelling but in a labelled with ^{15}N soil (indirect method).

The quantification of mineralized nitrogen by several methods showed that, if 75% of added nitrogen was mineralized using the direct method, only 60% of mineralized nitrogen was quantified by the difference method, and only 48% by the indirect method.

It seemed that nitrogen utilization coefficient according to the difference and A value methods risk to be lowered comparing to those obtained by the direct method, due to the interactions between the added and native soil nitrogen (Added Nitrogen Interactions). A simple method of calculation has been suggested in evaluating and better understanding the phenomenon of ANI. Its presence make uncertain the basic hypothesis used in the difference method and when the A value is determined.

A systematic use of treatment without N application is recommended in order to facilitate the interpretations of results.

EFFECT OF WATER STRESS ON NITROGEN-FIXATION OF TWO CULTIVARS OF COMMON BEAN (*PHASEOLUS VULGARIS* L.)

RAMOS, M.L.G.(1)(2), PARSONS, R.(2), JAMES, E.K.(2),
SPRENT, J.I. (2)
(1) Permanent address: EMGOPA/CNPq C.P. 49, Goiania (GO) 74210, Brasil.
(2) Department of Biological Sciences, University of Dundee, Dundee
DD1 4HN, UK.

An experiment was carried out to study the effect of water stress on nitrogen fixation and nodule structure of two cultivars of common bean (Carioca and EMGOPA-201). Seeds were inoculated at the time of planting with a mixture of two strains of *Rhizobium*. Twenty four days after planting, water was withheld from half of the plants; the other plants were kept as a control (watered). Plants were harvested after 5 and 8 d of water stress, and for each harvest there were control plants. In general, cv. Carioca was more affected by water stress then EMGOPA-201. Water stress decreased shoot dry weight (SDW), nodule dry weight (NDW) and specific nitrogenase activity (SNA). After 5 d water stress both cultivars had lower NDW then control. At 8 d water stress there were no differences among treatments. SDW was lower for Carioca at 5 d water stress and did not differ from control after 8 d stress. At 8 d water stress there were no differences between treatments or cultivars. At 5 d water stress SNA decreased in relation to the control. Bacteroid PHB content was greater in stressed than control nodules. Both cultivars had lower SNA after 8 d water stress. After 5 d stress infected cells of EMGOPA-201 were little affected, although there were some vacuolos in then. After 8 d stress peribacteroid membranes (PBMs) were ruptured and bacteroid membranes were damaged. With Carioca, damage to infected tissue was more pronounced, with host cell vacuolation and loss of PBMs at 5 d. At 8 d after stress, there was degradation of host cells cytoplasm and senescence of bacteroids, with their release into intercellular spaces. At 5 d after stress, cortical cells from nodules of both cultivars were no longer turgid, but appeared collapsed, with interlocking cell walls, and there was considerable deposition of darkly-staining material within the cells specially in cv. Carioca. Vascular bundles were also plasmolysed in appearance. The intensity of immunogold-labelling of intercellular cortical glycoprotein, with the monoclonal antibodies MAC 236/265, increased with stress treatments for Carioca, but EMGOPA-201 had high intensity of labelling in the control, decreased at 5 d water stress and it was not observed at 8 d water stress.

SYMBIOTIC PROPERTIES OF RHIZOBIA ISOLATED FROM THE ROOT NODULES OF LEGUMINOUS TREES

RÄSÄNEN L.A and LINDSTRÖM K.
Department of Applied Chemistry and Microbiology, POB 27,
FIN-00014 University of Helsinki, Finland

Acacia spp. and *Prosopis* spp. are leguminous trees, which grow in dry and semidry regions in the tropics. Many of these tree species are used in reforestration programmes. The aim of our study was to test the host specificity of three rhizobial strains isolated from nodules of *A. mollissima, A. senegal and P. chilensis*. The strains represent three different rhizobial groups (species). To get an overviw of the infection process between rhizobia and *Acacia* and *Prosopis* trees we investigated a) if the trees had root hairs, b) if deformation of root hairs occurred and c) if infection threads occurred.

Nodulation of *A. mellifera, A. nilotica, A. senegal, A. seyal, A. sieberana, A. tortilis (raddiana), P. cineraria, P. chilensis, P. juliflora and Sesbania rostrata* was tested with three rhizobial strain in greenhouse conditions. The length of shoots, acetylene reduction, shoot, root and nodule dry weights and nodule numbers were determined at harvest. All rhizobial strains induced nitrogen-fixing nodules within four or five weeks on all *Acacia* and *Prosopis* species tested, but not on *S. rostrata*. However, there were some differences between the strains in how they affected growth of different tree species.

For the infection study *Acacia* and *Prosopis* seedlings were grown in glass jars filled with a sterilized mixture of washed sand, Leca gravel and vermiculite.The roots of the plants were stained with 0.01 % methylene blue and examined under bright field microscopy. All tree species examined had root hairs, but they did not cover roots continuously, like in the case of temperate herbaceous leguminous plants. The root hairs were mainly located on lateral roots. The main root had root hairs on its younger parts.There were differences in the amounts of root hairs in different tree species. E.g. *A. seyal* only occasionally had short root hairs.

Root hairs of all species were deformed 7 days after inoculation. The typical feature for root hairs of *A. mellifera, A. nilotica, A. senegal and A. tortilis (raddiana)* was that they were short and thick. Similarly, their infection-thread-like structures were found mostly in short root hairs. *P. cineraria P. chilensis, P. juliflora* and *A. sieberana* had long curled root hairs and to a less extent short root hairs. "Traditonal" infection threads were common for these species.

BREEDING FOR NITROGEN FIXATION IN COMMON BEAN (*PHASEOLUS VULGARIS*), WITH SELECTION IN EARLY GENERATIONS.

RIBEIRO JUNIOR, W.Q.(1,2,3), SPRENT, J.I.(2), WAUGH, R.(3), POWELL, W.(3), BECK, D.(4), KIPE-NOLT J.(5), MUNOZ, E.(4), RAMOS, M.L.G.(1,2).
(1) EMGOPA/CNPq, C.P. 49 Goiania (GO) Brasil 74210, (2) Department of Biological Sciences, University of Dundee, Dundee DD1 4HN, UK,
(3) CIAT - Apto Aereo 6713, Cali, Colombia, (4) Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA. UK, (5) Hartline Science Center, Bloomsburg University, Bloomsburg, USA.

This project is designed to test the hypothesis that although biological nitrogen fixation (BNF) is a quantitative character with high environmental influence, selection in early generations can be more efficient than in late generations, due to the concentration of favourable genes. Initially, two genotypes (A295 and WBR 2255) were identified for a breeding project, considering not only BNF but also agronomic characteristics. Approximately 800 F2 seeds were obtained from a cross between these lines. Part of this material (687 genotypes) was selected in F2/F3 generations, for symbiotic capacity. As the evaluation was destructive in F2, the selected material had to be regenerated by embryo culture from immature seeds, allowing F4 seeds to be obtained. Because selection pressure was so strong by this stage, only 3% of the original number of families was represented. In parallel, the remaining genotypes, 113 families of the original 800, were used for Single Seed Descent (SSD), to the F4 generation. The best and worst genotypes were used to select molecular markers linked to BNF. For a number of traits indicative to BNF capacity, the early selected group showed better average BNF than the SSD group, where no selections have been conducted. This reinforces the idea that selection in early generations should be used in breeding projects for BNF improvement. However, the random group showed better Harvest Index, suggesting that this parameter must also be taken into account.

HIGH NODULATION VARIANTS WITHIN CHICKPEA CULTIVARS ARE ALSO HIGH FOR NITROGEN FIXATION.

RUPELA O.P.(1), DANSO S.K.A.(2) and JOHANSEN C.(1)
(1) Agronomy Division, ICRISAT Asia Center, Patancheru 502 324, Andhra Pradesh, India. (2) International Atomic Energy Agency, Wagramerstrasse 5, P.O. Box 100, A-1400, Vienna, Austria.

Natural occurrence of genetically determined nonnodulating (Nod$^-$), and low and high nodulating plants within chickpea (*Cicer arietinum* L., a self-pollinated grain legume) landraces and cultivars has been observed (Onkar Singh et al. 1992; Rupela 1992; Rupela and Johansen, unpublished). Occurrence of nodulation variants was also observed in 81 of 86 recently developed advanced breeding lines. Detailed studies have so far been restricted to nodulation variants from a few cultivars, including ICC4948 (=G130) and ICC5003 (=K850). In field experiments, high nodulation (HN) selections from these cultivars had 13-31% higher grain yields than the low nodulating (LN) selections of the same cultivar at low mineral N supply (O.P. Rupela and C. Johansen, unpublished). This is contrary to observations on the supernodulating soybean and common bean which invariably yielded lower than their normally nodulated parents (Buttery et al., 1990; Pracht et al., 1994). The HN, LN and the original varieties of these two cultivars comprised part of a field experiment aimed at quantification of N_2 fixed by these lines using a ^{15}N dilution method in the postrainy season at ICRISAT Asia Center in 1993/94. The high nodulating selections of the two cultivars fixed 33 to 50 kg N ha^{-1} which was 23-25% greater than their respective original varieties and 46-81% greater than their respective LN selections. Also, the proportion of fixed N_2 in the HN selections was 2-7% greater than their respective LN selections and their original varieties. Such aspects as exploiting rhizobia x host-plant interactions need to be reconsidered when plants of widely different nodulation capacities occur within a given cultivar.

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NITROGEN FIXATION BY DIFFERENT STRAINS OF *RHIZOBIUM GALEGAE.*

SARAND R., LAITAMM H., JAANISTE I.
Estonia Research Institute of Agriculture, Teaduse 1, Saku, Harju County
EE3400, Estonia

To increase the success of cultivation of leguminous plants, it is important to increase and stimulate in their rhizosphere the bacteria taxons, that form the most effective symbiosis between plants and bacteria.

Purpose of the investigation work is selection of most effective species-and sort specific nodule bacteria strains for Goat's rue (*Galega orientalis* Lam.) that would be durable and suitable in our climate conditions. Goat's rue, a very good protein and fodder producer, is naturally absent from Estonia field soils so it is to find out the most active nodule bacteria association for Goat's rue's raising sort "Gale" and use it in nodule bacteria preparation "Galeperliin", that is made in a Microbiology Laboratory of Estonian Research Institute.

Selection methods for estimating the effectiveness of symbiosis are Jenesen's tube method, where seedlings are grown up in phytotron and symbiotic effectiveness is measured on plants by using acetylene reduction test.

We have carried out a number of phytotrone experiments using Goat's rue's sort "Gale" and 10 different bacteria strains. One of our strains - *Rhizobia galegae* 740 - have got an author certificate in 1988, that was used in field experiments in 1985, about the effectiveness of nodule bacteria strains, based on the geographical network method. In phytotrone experiments we have also compared our strains with Swedish phytotrone experiments we have also compared our strains, based on geographical network method. In phytotrone experiments we have also compared our strains with Swedish strains of *Rhizobium galegae* and mesured their symbiotic effectiveness. Finally the activity of different bacteria strains is controlled in field experiments.

HOST CULTIVAR-SPECIFICITY OF THE SOYBEAN RHIZOBIA

SATO, T., MASUDA, Y.*,HIRANO, T., OHWADA, T. AND TAZAKI, H.
Department of Bioresource Chemistry, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080, Japan
*Yoshino Kogyosho, 2-6,Ojima 3, Koto-ku,Tokyo 136,Japan

Bradyrhizobium japonicum strain S 32 forms ineffective and small nodules on lateral roots of 4 nematode-resistant (NR) soybean cultivars. They also had a nitrogen fixing ability but very low compared with effective nodules formed on roots of 5 nematode-susceptible (NS) cultivars. The majority of proteins from cell free extracts of S 32 and OUG 117 (effective strain for both soybean groups), cultured with or without addition of NR soybean (cv. Toyosuzu) root exudate, migrated to the same positions in 2-dimensional electrophoretic gels. However, among them, quantities of 3 proteins were significantly increased in the cells cultured with root exudate of Toyosuzu (NR), compared with those without the root exudate. The objectives of this study are to clariy the mechanisms of host (cultivar)-specificity of the strain S32.

Among the amino acids in radicles of soybean cvs, amount of Asn was the highest (ca. 80%) followed by Ala, Ser,Val and Phe. Four kinds of polyamines (cadaverine-Cad, putrecine-Put, spermidine-Spd, and spermine-Spm) were detected in these radicles. Among them, Cad was the highest component, comprised ca. 90% of total. There could not find significant differences among the 2 groups from the data of amino acids and polyamines. Volatile components of the radicles of these cultivars were compared using ion-chromatographic patterns. Three componints out of about 40 detected by this method were selected from the results of stepwise descriminant analysis. These soybean cultivars were divided into 2 groups, NR- and NS-cultivars, using scatter plot of these 3 components. As the results of HPLC (Si, detection; 350 nm), a component which is assumed as terthiophene, was detected in alcohol extracted fraction of cv. Toyomusume (NR), but not in cv. Kitamishiro (NS).

INFLUENCE OF ACTIVE INGREDIENTS ON *RHIZOBIUM* - LEGUME DINITROGEN FIXATION.

SAWICKA, A.
Department of Soil Microbiology, Agricultural University of Poznan, Wolynska 35, 60-637 Poznan, Poland.

The legume plants, like peas, small beans, white lupine, yellow lupine and soybean - inoculated with active dinitrogen fixing bacteria - were cultivated on experimental plots. Two kinds of active substances were used against the weeds: imazethapyr (added to the soil after sowing and sprayed on the plants after shooting) and linuron (added to the soil after sowing). The experimental plots without herbicides were used as the control. The numbers of different microorganisms in the soil were aanalized during the vegetation season. Besides, dinitrogen fixation activity was measured twice directly on the experimental plots: at the beginning of plant flowering and during full flowering, with using acetylene to ethylene reduction method. The applied, in our experiment, active substances mostly inhibited *Rhizobium* - legume dinitrogen fixation. Imazethapyr sprayed on the plants resulted in most distinct decrease of N_2 - fixation by lupine symbiotic bacteria. The same substance applied to the soil resulted in increase of small beans dinitrogen fixation. It was not so distinct influence of these substances on number of microorganisms in the soil under plants.

GROWTH CHARACTERISTICS OF *AZOLLA-ANABAENA* SYMBIOSIS IN OSAKA, JAPAN

SHIOMI, N.(1), KITOH, S.(1), UHEDA, E.(1) AND WATANABE, I.(2)
(1) Research Institute for Advanced Science and Technology, University of Osaka Prefecture, Sakai 593, Osaka, Japan
(2) Faculty of Bioresources, Mie University, Tsu, Mie-ken, 514, Japan

Azolla is an aquatic fern which can grow without nitrogenous nutrient because of the N_2 fixing ability of symbiotic *Anabaena*. About 100 strains of *Azolla* composed of 7 species were collected mainly from IRRI (Philippines), amd their growth characteristics were examined in Osaka, Japan.

1. The growth of 50 *Azolla* strains was examined in the open. A good growth in average weight gain from April to November was observed in *A. mexicana* (2015, 2020), *A.caroliniana* (3011, 3022, 3025), *A.microphylla* (4072) and native *A.filiculoides* (1090). In mid summer most of the *Azolla* strains showed poor growth except *A.mexicana* (2015) and *A.caroliniana* (3001).
2. Nutrient removal capacities by 10 *Azolla* strains of batch culture were determined in a big pot (64x44x39 cm, 50 l) from the point of aquatic plant use in sewage treatment. *A.mexicana* (2025) showed the highest removal of nitrogen (189 mg/m^2/day) and phosphorus (54 mg/m^2/day) in May. *A.filiculoides* (1090) also showed a high removal capacity. In August *A.pinnata* (7004) showed high nutrients absorption (126 mg - N and 38 mg - P) and biomass. However, *A.nilotica* and *A.rubra* have shown poor growth and lower nutrient removal capacities in all seasons.
3. A high nitrogen level in the medium inhibited strongly both growth and N_2 fixation of *Azolla*. *Azolla* plants (117 strains of 7 species) were screened for tolerance to ammonium on the basis of their growth in the medium with or without ammonium. *A.nilotica* was the most sensitive species of all, followed by *A.rubra* whereas *A.pinnata* var. *pinnata* was the most tolerant one. Especially, *A.mexicana* (2007, 2019), *A.caroliniana* (3016), *A. microphylla* (4039, 4040, 4064, 4072) and *A.pinnata* (7002, 7004), which have grown in the medium with 20 mM of ammonium, showed about 90% growth of the control in the ammonium free medium. No tolerant strain in *A.filiculoides* was found as far as examined.
4. Recovery of gallium (Ga), which is an important metal in the advanced industry, was tried by the use of *Azolla*. *A.filiculoides* (1035) and *A.mexicana* (2011) showed a comparatively good growth and absorption of Ga below 13.08 ppm of Ga in the medium. *A. pinnata* var.*pinnata* (7002) was the most sensitive to Ga. Gallium was not incorporated into the symbiotic *Anabaena* in all strains examined. Recovery of Ga by three water plants (*Spirodela polyrhiza*, *Lemna minor*, *Lemna gibba*) has been examined and compared with that of *Azolla*. *L. minor* was the most tolerant to high Ga and the Ga concentration in the plants increased gradually with aging.

BREEDING SOYBEAN FOR IMPROVED NITROGEN FIXATION

SICHKAR V.I., PATYKA V.F., TOLKACHEV N.Z., KNYAZEV A.V.
Plant Breeding Institute, 3 Ovidiopol Road, Odessa, 270036, Ukraine.
Agricultural Institute, The Crimea Branch of Soil Microbiology, 107 Karl Marx Str. Gvardeyeskoe, Symferopol, the Republic of Crimea, 334080, Ukraine.

To start breeding soybean with the increasing ability to fix nitrogen, one has to find donorsand sources of the above trait. This feature is characteristic of the following varieties: Belosnezhka, Provar, Charivnytsya Stepu, Krepysh, Harosoy, Yug 40 and others. Second , one has to recombine the traits of the selected parents by means of cross-breeding and creaction of hybrid efficiency in plant-breeding for improving of nitrogen fixation. First, it is to hybridize donors and sources of high nitrogen fixation. To combine the improved nitrogen fixation and productivity in the same genotype, it is reasonable to hybridize a donor of nitrogen fixation and a high-yielding variety which is also well-adapted to certain climatic conditions.

For the results to be reliable, the experiment should be based on considerable quantities of hybrid plants. To our mind, the selection for nitrogen fixation should be started in F3 with 400-500 plants being checked in each combination. We just reject the plats which are of apparently little value as regards the feature in question. The final assessment is to be based on the indices for a number of generations.

Our plant-breeding program implies the assessment of 400-500 F3 plants of 4-5 hybrid populations under the conditions of a phytotron in the nitrogen-free medium in autumn and winter time. Then we select about 50% of F3 plants as regards their above the ground mass, the content of protein in it and the seed productivity. Those plants are propagated in families in the field, assessed in a phytotron in the nitrogen-free medium again and shown in the field with replications. The results obtained enable a plant breeder to more precisely identify the lines of F5 which are better as regards their nitrogen fixation ability. In F6-F8, the above cycle is repeated, the best lines of F5 being used as the initial material.

EFFECTS OF DROUGHT AND SALT STRESSES ON NITROGEN FIXATION OF MIMOSA TREE

SONG, S-D. AND KIM, Y-M.
Department of Biology, Kyungpook National University, Taegu 702-701, Korea

Mimosa tree (*Albizzia julibrissim*), a nodule bearing N-fixing woody legume, is an important vegetation component of the temperate deciduous forest in Korea and known to promote soil fertility and succession process of less productive areas. Plants grown in the natural communities showed the maximum nitrogenase activity of 20.8 μmol C_2H_4 g fr wt nodule^{-1} h^{-1} in May and the activity decrease of 80% during dry and hot season of July and August then a 2nd peak of activity in September and October. Seedlings formed nodules and showed the nitrogenase activity after 4 weeks of germination and the maximum rate of 45 μmol C_2H_4 g fr wt nodule^{-1} h^{-1} in 3 months then maintained 20 μmol C_2H_4 g fr wt nodule^{-1} h^{-1} in 5 months. Nodule, leaf and stem/root showed 6.5%, 4.2% and 2.0% of nitrogen content on dry weight basis, respectively. Restraint of water in the rhizosphere for 3 and 7 days during the early growth resulted in 40% and 90% decreases of soil water content, -1.0 MPa and -2.3 MPa of leaf water potential, 60% and 80% decreases of acetylene reduction activity of nodule, respectively. The relative plant growth of 62, 82 and 100% was attained by the water supply of 20, 50 and 100 mL per pot, respectively during the early growth. Salt treatments with 25, 50 and 100 mM NaCl resulted in the relative nitrogenase activity of 77, 50 and 25% compared to the control plant in 6 days and detected no activity by 50 and 100 mM NaCl treatments in 28 days during later growth. Nodules treated directly with 0, 25, 50. 100 and 200 mM NaCl showed the relative nitrogenase activity of 100, 58, 37, 30 and 3%, respectively. Salt stress caused no changes of leaf water content in the early growth, but showed significant inhibition by 3 weeks of treatment during the later growth. Diurnal changes of nodule activity showed 2 peaks at 10 AM and 10 PM in the later growth. Seedlings grown in the field and the growth chamber showed the nitrogenase activities of 210 and 130 μmol C_2H_4 g fr wt nodule^{-1} h^{-1} for the annual plant and 70 μmol C_2H_4 g fr wt nodule^{-1} h^{-1} for the biennial plant until 4 hours. Temperature gradients showed the maximum nodule activity at 30 ℃ and the plants raised in the growth chamber showed 54% and 32% decreases of the activity, respectively for annual and biennial plants compared to the control.

MIXED INOCULUM DEVELOPMENT FOR AFRICAN ACACIAS

SUTHERLAND, J.M.(1), ODEE, D.W.(2), MCINROY, S.G.(1), KIMITI, J.(2), MACHUA, J.(2), MULUVI, G.(2), STANFORTH, A.(1), SPRENT, J.I.(1)
(1) Department of Biological Sciences, University of Dundee, Dundee, DD1 4HN, UK
(2) Kenya Forestry Research Institute, P O Box 20412, Nairobi, Kenya

A series of experiments was conducted to test the efficacy of mixed rhizobial inocula on the nodulation and growth of seven African acacia species/subspecies. Experiments were conducted in a glasshouse in Dundee at two nursery sites in Kenya in both sterile and unsterile soil. The purpose of this strategy is to reduce both labour inputs and potential errors in setting up progeny testing trials using specific single strains. The project is linked to germplasm collection and evaluation of African acacias being conducted by R D Barnes and C W Fagg, Oxford Forestry Institute. Both projects are funded by the Overseas Development Administration, UK.

Species tested were: *Faidherbia albida; Acacia arenaria; A. karroo; A. nilotica* ssp. *kraussiana; A. tortilis* ssp. *heteracantha; A. tortilis* ssp. *spirocarpa; A. senegal.* Mixed inoculum experiments using 8 - 10 rhizobial strains were conducted at two sites in Kenya. Rhizobia were recovered from one of these experiments and capillary electrophoresis protein profiles were compared to identify the most competitive strains. In another experiment the mixed inoculum was assessed in both sterile and unsterile conditions by comparison with homologous single strains. Since acacias are genetically very variable the relative enrichment of natural abundance ^{15}N in nodules was also used to assess strain effectiveness in individual plants. *A. tortilis* ssp. *heteracantha* exhibited the greatest range of strain effectiveness by this assessment.

The principle findings from these experiments are as follows. 1. Acacias vary widely in their specificity for rhizobial strains with *Faidherbia albida* being very promiscuous and *A. tortilis* ssp. *heteracantha* recalcitrant. There were site-species interactions for nodulation characteristics but dry weight was greater at the hotter, sunnier site. 3. Growth was greater in unsterile than in sterile soil. 4. Dry weight was significantly increased in five of the seven spp/ssp with a mixed inoculum. In a sixth species, *A. nilotica* ssp. *kraussiana* dry weight was significantly increased using a single strain inoculum in unsterile conditions while in the seventh species, *A. arenaria* the single inoculant strain increased dry weight only in sterile soil.

The general conclusion from this work is that the strategy of using mixed inocula may be preferable to single strain inocula particularly with inherently unstable strains.

IDENTIFICATION OF *RHIZOBIUM* BY USE OF REP-PCR IN WHITE CLOVER NODULES

SVENNING M.M., JOHANNESSEN M., SOLHEIM B. and BHUVANESWARI T.V.
Department of Plant Physiology and Microbiology, IBG, University of Tromsø, 9037 Tromsø, Norway.

The nitrogen-fixing symbiosis between white clover and *Rhizobium leguminosarum* biovar *trifolii* is important in the temperate zones of the world. The host plant is normally well nodulated in the field by the indigenous *Rhizobium* population. The genetic diversity of rhizobia is quite considerable. The possibility of analysing the diversity in natural populations of *Rhizobium* has improved during the last years by the use of specific DNA sequences to fingerprint the bacterial genomes (de Bruijn 1992, Versalovic *et al.* 1991).

We have used the REP-and ERIC-primers in the PCR method to examine identity and diversity of isolates from indigenous *Rhizobium* populations nodulating different genotypes of white clover. In this study we present the usefulness of this method to fingerprint *Rhizobium* from cell cultures as well as *Rhizobium* in nodules. These studies are part of a research program at the University of Tromsø on the white clover- *R. l.* bv. *trifolii* interactions in a cold temperate climate. Topics we focus on are:

- Genetic and phenotypic diversity in the indigenous *R. l.* bv. *trifolii* population
- Diversity in the *R. l.* bv. *trifolii* population nodulating various genotypes of white clover during subsequent growing seasons
- *R. l.* bv. *trifolii* - white clover interactions at low temperatures

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ISOLATION AND APPLICATION OF DNA PROBES AND PCR PRIMERS FOR IDENTIFICATION OF *RHIZOBIUM GALEGAE*

TAS E.(1), LEINONEN P.(2), SAANO A.(1), PIIPPOLA S.(1), LINDSTROM K.(1).
(1) Department of Applied Chemistry and Microbiology, POB 27, FIN-00014, University of Helsinki, Finland (2) Elomestari Ltd., Fin-51900 Juva, Finland

A *Rhizobium galegae* HAMBI 1174 strain-specific probe (P3; 342 bp) was isolated by subtraction hybridization (Tas et al.,1994), and a *R.galegae* species-specific probe (P09; 900bp) was identified in the putative host-specificity gene region of *R.galegae* HAMBI 1174. The specificity of both fragments was proved in dot blot and Southern blot hybridizations. PCR primers were designed based on the sequences of these specific fragments. The two fragments could be concurrently amplified from strain HAMBI 1174. The species-specific fragment was amplified from all *R.galegae* strains in our collection. Interestingly, the restriction pattern of this 900 bp fragment showed slight differences between the strains from the two different plant hosts. No amplification was detected from other rhizobia or other bacterial species. Dot blot hybridization and PCR could be used for identification of strais directly from soil and nodule samples (Tas et al.,1995). To elucidate the functions of the genomic regions from which these fragments are derived from, a 1.1 kb region involving P3, and the 900 bp cloned species-specific fragment were sequenced. No significant homology was found to any known genes recorded in date bases. Further analysis of the DNA sequences, and Northern-hybridization tests on whether these putative gene regions are induced during nodulation are among the aims of our future studies.

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APPLICATION OF PRIMER-BASED TECHNOLOGY TO FINGERPRINT THE GENOMES OF *BRADYRHIZOBIUM* SPP. USED IN THAILAND

TEAUMROONG, N.(1), BOONKERD, N.(1) and MUROOKA, Y.(2)
(1) Institute of Agricultural Technology, School of Biotechnology, Suranaree University of Technology, Nakhon-Ratchasima 30000, Thailand
(2) Department of Fermentation Technology, Faculty of Engineering, Hiroshima University, Japan

Total chromosomal DNAs were isolated from *Bradyrhizobium* spp., 10 from *Vigna radiata* and the rest from *Arachis hypogaea*. These DNA served as templates with REP, ERIC and RAPD primers in the PCR analyses. Using PCR patterns to differentiate the ten strains from *V.radiata* with primers REP, ERIC and RAPD, they can be grouped into 7,6 and 6 different. However, PCR patterns generated from ERIC and RAPD primers showed that strains E-7-1 and E-17-1 have the same pattern while the rest are different.

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GENETIC DIVERSITY AND PHYLOGENY OF RHIZOBIA NODULATING *MEDICAGO RUTHENICA* (L.) LEDEB

VAN BERKUM, P.[1], BEYENE, D.[2], BAO, G.[3], CAMPBELL, T.A.[1], AND EARDLEY, B.D.[4]
[1] Soybean and Alfalfa Res. Lab, USDA, ARS, Beltsville, MD 20705, USA;
[2] Inst. of Agricultural Res., POB 2003, Addis Adeba, Ethiopia;
[3] Grassland Res. Inst., CAAS, Huhehot, PR China;
[4] Inst. of Molecular Evolutionary Genetics, Penn State Univ. Berks Campus, Reading PA 19610, USA.

Although recognized as having agronomic potential comparable to cultivated alfalfa (*Medicago sativa*), *M. ruthenica* still needs to be developed as a crop plant. Seeds from 101 plant populations were collected from three ecologically diverse sites in Inner Mongolia, which is the probable center of origin of *M. ruthenica*. The three sites also yielded 104 isolates of *Rhizobium*, which were evaluated for nodulation and effectiveness for nitrogen fixation to identify potential inoculant quality strains. Variability among the strains was determined to ensure the maintenance of a diverse population. Fifteen strains, selected on the basis of diversity, were moderately to highly effective for nitrogen fixation on the original trap host plant. Characterization of 68 strains indicated wide phenotypic diversity and little similarity to the taxonomic reference strains of *Rhizobium* included in the analysis. Variability among the strains also was detected with PCR analysis using primers for repetitive extragenic palindromic (REP) sequences and, with the exception of one strain, no relationship with *S. meliloti* was detected by clustering analysis of the data. A few highly divergent lineages and a large cluster of closely related electrophoretic types (ET) were identified among 99 strains comprizing 61 ET's based on multilocus gel electrophoresis of 12 loci. The phylogenetic relationship of the *M. ruthenica* symbionts with related taxa within the alpha-2 subgroup of the *Proteobacteria* was determined from 16S rDNA sequencing analysis. A distance matrix using the model of nucleotide substitution of Jukes-Cantor was computed and was used to obtain an unrooted tree using the Fitch-Margoliash method. *Rhizobium spp.* of *M. ruthenica* formed a cluster, which branched from the tree between the branch points of *R. etli* and *R. tropici*.

THE GROUNDNUT-*BRADYRHIZOBIUM* SYMBIOSIS: EFFECTS OF INOCULANT STRAINS AND SOIL ACIDITY

VAN ROSSUM D. (1), BOOGERD F.C. (1), MYOTCHA A. (2), DE HOOP B.M. (3), VAN VERSEVELD H.W. (1), and STOUTHAMER A.H. (1)
(1) Vrije Universiteit, Dept. Microbiology, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands; (2) Soil Productivity Research Laboratory, Private Bag 3757, Marondera, Zimbabwe; (3) Agricultural University, Dept. Mol. Biol., Dreyenlaan 3, 6703 HA Wageningen, The Netherlands.

This study aimed at identifiying *Bradyrhizobium* strains that can enhance groundnut productivity on peasant farms in Zimbabwe. To this end, a large number of *Bradyrhizobium* strains were tested for their symbiotic performance with four groundnut (*Arachis hypogaea* L. spp. *fastigiata*) cultivars. Another experiment was performed to assess the effect of soil acidity. All experiments were performed in potted soil in a greenhouse in Zimbabwe.

Firstly, many parameters were assessed for their effect on seed (groundnut kernel) yield. Leaf area showed a significant positive correlation with seed yield, while nodule dry weight did not correlate. It was concluded that photosynthesis, rather than nitrogen fixation, limits productivity.

The main effect upon inoculation with various *Bradyrhizobium* strains concerned the infectivity: different strains caused significantly different amounts of nodule tissue on the groundnut roots. This did, however, not lead to differences in the amounts of nitrogen accumulated in the plant.

Of the four groundnut cultivars tested at neutral pH, cultivar 'Natal Common' performed superiorly; the two cultivars tested at acid pH showed that 'Falcon' outperformed 'Plover'.

Soil acidity negatively affected plant development (leaf area; plant dry weight), while nodulation was enhanced (nodule number; nodule dry weight). This acidity-enhanced nodulation was most pronounced with indigenous *Bradyrhizobium* strains. Some of the inoculated strains were totally ineffective under acidity stress, while others performed well (MAR1576, MAR 1555, and MAR1510). Strain performance was found to be dependent on soil pH.

Nitrogen fixation was found to lead to soil acidification, while nitrate assimilation lead to alkalinisation.

PENTAPHASIC TAXONOMICAL STUDY OF *BRADYRHIZOBIUM* STRAINS NODULATING GROUNDNUT (*ARACHIS HYPOGAEA* L.).

VAN ROSSUM D. (1), SCHUURMANS F.P. (1), GILLES M. (2), MYOTCHA A. (3), VAN VERSEVELD H.W. (1), STOUTHAMER A.H. (1), and BOOGERD F.C. (1)
(1) Vrije Universiteit, Dept. Microbiology, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands; (2) Universiteit Gent, Dept. Microbiology, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium; (3) Soil Productivity Research Laboratory, Private Bag 3757, Marondera, Zimbabwe

This study aimed at identifying molecular and physiological markers for *Bradyrhizobium* sp. (*Arachis*) strains. The data obtained in this study were then used for taxonomic purposes, with a view to testing the consistency of similarity groupings across various methods.

To this end, seventeen *Bradyrhizobium* spp. strains and one *Azorhizobium* strain were compared using five genetic and phenetic features:

(1) partial (264 nucleotides) sequence analysis of the 16S rRNA gene (rDNA).
(2) amplified fragment length polymorphisms (AFLP, also RAPD) using 3 oligonucleotide primers yielding 42 markers.
(3) fingerprints of total cellular protein using SDS-PAGE.
(4) utilisation of 21 aliphatic and 22 aromatic substrates in auxanographic ò fixed-concentration plates.
(5) level of intrinsic resistance to 7 antibiotics.

Partial 16S rDNA analysis revealed the presence of 2 rDNA-homology groups amongst the seventeen *Bradyrhizobium* strains. A dissimilarity dendrogram, also comprising other organisms' rDNA data, showed that *Bradyrhizobium* spp. strains form a tight cluster with *Rhodopseudomonas palustris*, *Nitrobacter* species, *Afipia* species and *Blastobacter denitrificans*, but were only distantly related to other α-*Proteobacteria*, including other members of the *Rhizobiaceae* family.

Clustering the *Bradyrhizobium* spp. strains for their AFLP profiles, protein profiles and substrate utilisation revealed more diversity than rDNA analysis. Based on these 3 methods the 2 rDNA homology groups generally clustered separately, but there were always exceptions. It therefore appeared that a high rDNA similarity is a prerequisite, but not a guarantee, for high similarity values between AFLP profiles, protein profiles and substrate utilisation.

Intrinsic antibiotic resistance patterns and AFLP fingerprints were strain-specific and could therefore not be clustered or only at very low similarity levels.

The relationships, coming forth from each of the studied features, often had low compatibilities, casting doubt on the usefulness of a polyphasic approach in rhizobial taxonomy.

Van Rossum D et al (1995) Appl. Environ. Microbiol. 61, 1599-1609.

HOST RANGE STUDIES OF RHIZOBIA FROM AFRICAN *ACACIA* TREES

WANG. W., WHITTY,P. AND SPRENT J.I.
Department of Biological Sciences, University of Dundee, Dundee
Scotland, DD1 4HN, U.K.

We have isolated a number of fast-growing rhizobia strains from different African *Acacia* trees. Nodulation tests on plants revealed the nodulation ability of 15 rhizobia strains on 8 *Acacia* species and the specificity of host range has been determined. Strains having wide or narrow host ranges were found.

Plasmid detection tests were carried out which demonstrated these strains carrying several low copy mega-plasmids. Ten random oligonucleotide primers have been used for RAPD analysis on 15 different rhizobial strains from different *Acacia* trees. Greater variation was observed between these strains.

ELISA was carried out on the same strains and 7 additional strains and there was distinct differences between wide/narrow host strains and the addtional strains analyzed. Tri-parental mating and LPS-SDS PAGE showed that the mega-plasmid is very important for nodulation and there was clear differences between transconjugants and the recipient strains in there LPS types.

We are using a *nod*D probe and PCR of the *nod*H to identify differences between narrow and wide host range rhizobia.

FIELD AND POT EXPERIMENTS ON THE INFLUENCE OF DIFFERENT N DELIVERY POTENTIAL OF SOIL ON SYMBIOTIC N_2 FIXATION OF RED CLOVER

WERNER W. and LOPOTZ H.
Institute of Agricultural Chemistry, University of Bonn, Meckenheimer Allee 176, D-53115 Bonn, Germany.

It is well known that mineral N fertilization may reduce symbiotic N_2-fixation (BNF). However information is lacking whether different nitrogen concentration of the soil solution, resulting from a different N delivery potential may already have an impact on BNF. For this reason a field experiment with red clove was established on plots with different long-term organic fertilization which led to different total N contents (0.085 to 0.12%) and N delivery. Furthermore a pot experiment was conducted, where on the luvisol with 0.085% N_t different small amounts of N were continuously applied during the growing season to simulate a different N delivery potential. The field plots as well as the different treatments in the pot experiment were labelled with small amounts of $(^{15}NH_4)_2SO_4$ to determine the BNF by the isotope dilution technique.

In the field experiment the proportion of BNF of N accumulation decreased from 84 to 70% with increasing N delivery.

In the pot experiment the proportion of BNF of the total N accumulation decreased from 94% without additional N delivery to 83% in the treatment with the highest N delivery rate.

In red clover-ryegrass mixtures N from delivery was competitively taken up by the ryegrass, by that eliminating the negative effect of nitrogen in soil solution on BNF.

THE EFFECTIVENESS TEST OF *RHIZOBIUM* ISOLATES ON NITROGEN FIXATION IN ASSOCIATION WITH TROPICAL SHRUB LEGUMES

WIDJAJANTO D.W.(1), NURWANTORO (1), YUDIARTI T.(2)
(1) Faculty of Animal Sciences and (2) Research Center, Diponegoro University, Hayam Wuruk 4A Semarang 50224, Indonesia.

Tropical shrub legumes as *Leucaena leucocephala, Calliandra calothyrsus* and *Sesbania gladiflora* have a good potential in maintaining soil nitrogen availability. For example, nitrogen fixation contributes around 65% of N uptake by *Leucaena leucocephala* . Unfortunately, there are many factors influencing the nitrogen fixation. One of these are *Rhizobium* bacteria.

Research was aimed to find the effective *Rhizobium* bacteria which associated with tropical tree legumes. These were *Leucaena leucocephala, Calliandra calothyrsus* and *Sesbania glandiflora.*

Rhizobium isolates were tested using Siratro (*Macroptilium artropurpureum*) with modified Leonard Jar method. Crops were harvested at the end of growth period. These were separated into plant material and nodules. Plant material was dried in order to evaluate dry matter, crude protein and crude fiber content. Nodules were separated from root and analysed in order to determine the nitrogenase activity using Acetylene Reduction Assay (ARA).

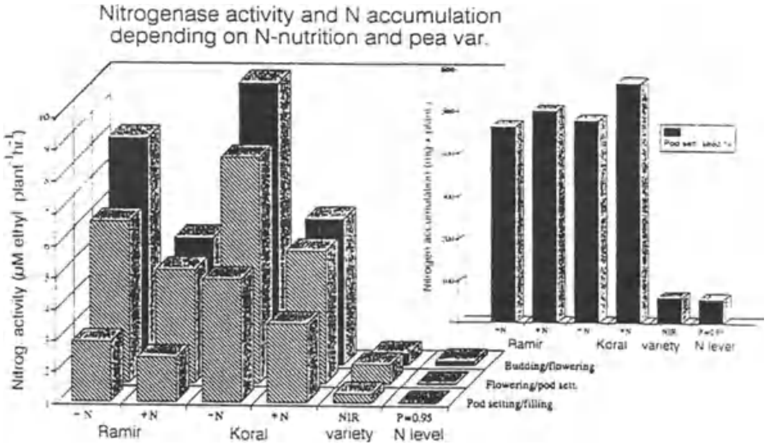
Siratro produced dry matter (DM), crude protein (CP) and crude fiber (CF) higher at all treated stands that control. Siratro inoculated with *Rhizobium* inoculant which is isolated from nodules of *Calliandra calothyrsus, Leucaena leucocephala* and *Sesbania glandiflora* produced amount of 7.27 g DM, 0.83 g CP and 2.45 g CF; 6.24 g DM, 0.70 g CP and 1.91 g CF ; 4.78 g DM, 0.64 g CP and 1.49g CF, respectively. While control produced 2.72 g DM, 0.43 g CP and 0.82 g CF. These formed 5, 4 and 2 nodules and resulted nitrogenase activity as much of 0.35%; 0.31% and 0.30%, respectively.

The experiment proved than all of isolates were effective in nitrogen fixation. These however have to be tested ti other limiting factors such as nutrients constitute, soil moisture and soil acidity in order to find out the effective *Rhizobium* isolates.

NITROGEN FIXATION AND ACCUMULATION DEPENDING ON PLANT VARIETY AND NITROGEN NUTRITION

WOJCIESKA, U., PODLESNA, A., MROCZKOWSKI, W., KOCON, A
Department of Plant Physiology, Institute of Soil Science and Plant Cultivation, Pulawy, Poland

The aim of this work was to study the effect of nitrogen nutrition on nodulation, nitrogenase and nitrate reductase activity as well as nitrogen accumulation in two pea varieties - Koral and Ramir. Plants were grown in pots with 1:1 soil - sand mixture, supplemented with appropriate amounts of nutrient components. A part of plants utilized mainly molecular nitrogen (-N), another received 115 mg N/kg of medium (+N), as ammonium nitrate. The varieties were found to differ from each other with respect to the investifated parameters. Koral had a higher nitrogenase activity than Ramir. Nitrate reductase (NR) activity was higher in the leaves of the Ramir than of Koral at both nitrogen levels. Ammonium nitrate decreased nodulation and nitrogenase activity in root nodules of both varieties (especially at the beginning of florescence) and decreased NR activity . At later development stages the inibitory effect of mineral nitrogen became less pronounced. The amounts of nitrogen accumulated by the plants were similar irrespective of nitrogen level in the environment, which indicated that weakening of molecular nitrogen fixation by ammonium nitrate was compensated by increased uptake of mineral nitrogen. Koral variety fixed N_2 better than Ramir, but Ramir assimilated more mineral nitrogen. It is supposed that nitrate reductase activity in the leaf tissue of pea plants can be an indicator of its reaction to mineral nitrogen nutrition.



NITROGEN FIXING BACTERIA FROM RHIZOSPHERE OF IRANIAN AND HUNGARIAN GRAMINEOUS PLANTS

AFSHARIAN M., KARPATI E. and SIK T. Department of Biotechnology, Godollo University of Agricultural Sciences, H-2103 Godollo, Pob 303, Hungary

Nitrogen fixing bacteria were isolated from rhizosphere of different Iranian gramineous plants (wheat, corn sugar beet, sorghum, Digitaria, Lolium and Dactylis) and two Hungarian rice cultivars using nitrogen free semisolid medium.

Isolates were characterized by colony morphology, growth on different carbon sources, oxygen sensitivity, nitrogenase activity and plasmid profiles.

Differences in growth on various carbon sources were observed. Both aerobic, microaerophilic and anaerobic pattern of growth were found among the isolates. Nitrogenase activities on malate and glucose as sole carbon source were varied.

Plasmid patterns were detected by pulsed field gel elected by pulsed field gel electrophoresis (PFGE) and compared.

NEW SYNTHETIC AND MULTI-SPECIES BACTERIAL INOCULANTS FOR PLANT GROWTH-PROMOTING RHIZOBACTERIA.

BASHAN Y., CARRILLO A., HOLGUIN G.
Department of Microbiology, The Center for Biological Research (CIB),
La Paz, P.O. Box 128, B.C.S., 23000 Mexico

Peat formulations are the carrier of choice for most bacterial inoculants. Although these inoculants have proven abilities, they also have some practical deficiencies which are difficult to overcome; therefore, new formulations are slowly being introduced using unconventional synthetic polymers like alginate which offer substantial advantages over peat. In the field of inoculation technology, the encapsulation of microorganisms is only experimental. These formulations encapsulate the living cells, protect the microorganisms against many environmental stresses and release them to the soil gradually when the polymers are degraded by soil microorganisms. They can be dry stored at ambient temperatures for prolonged periods, offer a consistent batch quality, a better defined environment for the bacteria, and can be manipulated easily according to the needs of specific bacteria. These inoculants can be amended with nutrients to improve the short-term survival of the bacteria upon inoculation. However, they are rather expensive relative to peat-based inoculants and require more bio-technical handling by the industry.

Alginate is the most common material for encapsulation. The preparation of macro-beads (1-3 mm in diameter) and microbeads (100-200 µm) containing bacteria is relatively easy and involves multi-step procedures.

Several Plant Growth-promoting Rhizobacteria, like *Azospirillum,* have erratic and unpredictable behavior under field conditions making them unlikely candidates for commercialization. For a better exploitation of their potential, we are proposing a new concept: mixed inoculation of more than one microorganism in a single carrier, inoculated simultaneously. The beneficial effect on plants is the synergistic effect of both (or several) microorganisms. In this inoculation type, *Azospirillum,* for example, may take the role of a "helper" bacteria or be "helped" by other bacteria to overcome its own unpredictability. In this regard, several synergistic combinations of *Azospirillum* and other microorganisms are known. Cellulose breakdown by *Cellulomonas* and pectin degradation by *Bacillus* were enhanced when co-cultured with *Azospirillum.* Synergism in nitrogen fixation occurred when cultures of *Azospirillum* were mixed with *Arthrobacter*or *Staphyloccocus.* Nodulation of legumes or infections with VAN fungi were increased when mixed or co-inoculated with *Azospirillum.* However, thus far, mixed inoculation is at the far end of contemporary inoculation research and development.

SURVIVAL OF ASSOCIATIVE BACTERIA ON ROOTS AND EFFICIENCY OF INOCULATION UNDER VARIOUS ENVIRONMENTAL CONDITIONS.

BELIMOV A.A. (1), KUNAKOVA A.M. (1), KHAMOVA O.F.(2), POSTAVSKAYA S.M. (2), SOKOVA S.A.(3), GRUZDEVA E.V.(1), VOROBYOV N.I.(1), KOZH EMYAKOV A.P. (1)
(1) Research Institute for Agricultural Microbiology, Podbelsky Sh.3, Pushkin-8, Saint-Petersburg, 189620, Russia.
(2) Siberian Institute for Agriculture, Ave. Korolev 2, Omsk-12, 644012, Russia.
(3) Moldavian Research Institute for Vegetable Growing, Ave. mira 50, Tiraspol, 278000, Moldavia.

Plant response to inoculation and population dynamics of the introduced bacteria (*Azospirillum lipoferum 137, Arthrobacter mysorens 7, Agrobacterium radiobacter 10, Flavobacterium sp. L30*) in the rhizoplane of barley, wheat, rice, oat, ryegrass, rape, Pe-tsai cabbage, tomatoes and alfalfa were studied in pot and field experiments. All of the strains colonized actively the rhizoplane of various plants and were generally detected throughout the experiments. The total counts of introduced bacteria on roots varied depending on plant genotype. However, differences in colonization ability and population dynamics of the bacteria among plant species in many instances were not significant. The number of *A.mysorens 7* and *A.radiobacter 10* was always higher than that of *A.lipoferum 137* and *Flavobacterium sp L30*. The overall pictures of survival rates of the strains in soil and in rhizoplane were very similar. The effect of "homologous" plant species (i.e. from the rhizosphere of which the strain had been initially isolated) on survival of the bacteria was not reliable. Therefore, the influence of plants on survival of the bacteria was not specific and the plants had very low capability for controlling a number of introduced bacteria in the rhizoplane. The beneficial effect of inoculation on plant development was very unstable and could be observed only in individual cases. In many instances the response of plants to inoculation with *Flavobacterium sp. L30* was the most conspicuous. No correlation between plant respones to inoculation and population size of the introduced bacteria or the origin of their initial isolation were established.

Pot experiments with barley and wheat grown under optimum and extreme environmental condions were conducted. The population size of the introduced bacteria varied significantly depending on soil moisture and their maximum count was observed at water shortage. To a less degree survival of the bacteria depended on soil temperature, acidity, soil type and nitrogen status of plants. The benefical effect of inoculation on barley yield was a maximum when the plants were grown under unfavourable conditions of soil moisture, acidity and temperature. Under stress conditions the free proline content in the inoculated plants was significantly less as compared with the uninoculated control. It is suggested that inoculation enhances a tolerance of plants for stresses.

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GENETIC RELATIONSHIPS AMONG ISOLATES OF *ACETOBACTER DIAZOTROPHICUS* RECOVERED FROM DIFFERENT HOSTS.

CABALLERO-MELLADO, J.[1], FUENTES-RAMIREZ, L.E.[1], REIS, V.M.[2] AND MARTINEZ-ROMERO, E.[1].
[1]Centro De Investigacion Sobre Fijacion De Nitrogeno. U.N.A.M. AP. P. 565-A, Cuernavaca, Mor. Mexico. [2]Embrapa KM 47, Seropedica 23851-970, Rio De Janeiro, Brazil.

A. diazotrophicus is a N_2-fixing bacterium which has been isolated from sugarcane, sweet potato and *Pennisetum purpureum* var. Cameroon, as well as from mealybugs associated with sugarcane plants. Previously, we reported (Caballero-Mellado, Martínez-Romero, 1994) a limited genetic diversity among isolates of *A. diazotrophicus* recovered from sugarcane plants. In this work, we report the genetic relatedness among isolates recovered from the different known host species obtained mainly from Mexico and Brazil.

The genetic relatedness of the isolates was determined by multilocus enzyme electrophoresis. A total of 7 distinctive electrophoretic types (ETs) were identified among 55 isolates. 35 of them were identical, corresponding to the ET 1, and 4 ETs were represented by 1 or 2 isolates only. Six ETs (ET 1 to ET 6) were closely related, forming a cluster at a genetic distance of 0.195 and exhibiting high DNA-DNA homology with reference strain PAl 5^T. A second line (ET 7), which contained six strains, was largely divergent and it was separated at a genetic distance of 0.53, and had only 54% DNA homology to the reference strain. These data suggest that the strains represented by the ET 7 could represent a distinct nitrogen-fixing species of the genus *Acetobacter*.

The analysis showed that isolates of *A. diazotrophicus* recovered from many different sugarcane varieties cultivated in geographic regions of Mexico, as well as from mealybugs associated to sugar cane plants belong to a single clonal lineage (ET 1). In contrast, isolates recovered from Brazilian sugarcane varieties and mealybugs associated corresponded to 7 different lineages, including the ET 1. These results may be related to the very different N fertilization levels that are applied in these two countries. The results clearly demostrated that the ET 1 was extensively distributed among all the hosts species analyzed. It will be important to determine if the strains represented by the highly predominant ET 1 could be more efficient on the growth promotion of the host plants, either by the involvement of indoleacetic acid (Fuentes-Ramírez, 1993), or by supplying nitrogen (Boddey et al. 1991) or by both, in comparison to other lineages.

Boddey RM et al. (1991) Plant and Soil 137,111-117.
Caballero-Mellado J, Martínez-Romero E (1994) Appl. Environ. Microbiol. 60,1532
Fuentes-Ramírez LE (1993) Plant and Soil 154,145-150.

ASSOCIATIVE SYMBIOSIS OF SOIL DIAZOTROPHIC BACTERIA AND PLANTS AND ITS CONTRIBUTION TO VEGETABLE YIELDS

EMTSEV, V.T., SELITSKAJA, O.V., PETROV-SPIRIDONOV, A.A., BRUK, M.H., KUBAREVA, O.G.
Department of Microbiology, Timirjasev Agricultural Academy, 127550 Moscow, Russia

We have studied associative diazotrophs isolated from vegetable crops under sheltered ground conditions, their nitrogenase activity and influence on plant growth and development. In the rhizosphere of cucumbers the nitrogen fixing activity is realized mainly through facultative anaerobic diazotrophs, among which *Klebsiella, Enterobacter* and *Bacillus* strains dominate. Among the isolated representatives of *Klebsiella*, one strain (TSHA-91) was identified as *K. planticola*. It was characterized by a high nitrogen fixing activity and ability to inhibit growth of phytopathogenic fungi. Inoculation with *K. planticola* TSHA-91 strain increased the yields of cucumber by 21-23%, tomato - by 31%, potato - by 21% and pumpkins - by 31%. An ultimate aim of our studies is to create a highly effective biofertilizer for nonleguminous plants which could at least partly substitute for application of mineral nitrogen thus ensuring ecologically safe crop production under conditions of sustainable agriculture.

RHIZOTRONE FOR OBSERVATIONS OF NITROGEN FIXATION BY SOIL BACTERIA

ERMAKOV E.I., ZHELTOV YU.I., STEPANOVA O.A. Agrophysical Institute, 14 Grazhdansky, St.Petersburg, Russia

The rhizotron ROST-4M with the film porous root medium is attendand for investigating the growth dynamics of root systems and attendand microorganisms in the plant ontogeny. The rhizotron contains four independent blocks with the porous film medium vertically disposed on the rigid base, on the surface of which plant roots are arranged in a natural way. Each of four media has the width of 50 and the height of 100 cm. Rhizotron blocks are provided with the individual system of metering feeding the nutrient solution into the root medium. The rhizotron has the irradiation modules and the necessary equipment for protecting roots from light and mechanical influences. The automatic maintenance of programmed conditions of the plant vital support throughout the ontogeny is also provided in the rhizotron. The root medium is the thin layer porous superdense, chemically inert biologically resistant material through capillaries of which the nutrient solution runs continuously from above downwards in the recirculation regime. The film root medium due to some characteristics is a physical soil model permitting to investigate root systems and the complex of attendant microorganisms under conditions approximated mostly to the natural ones.

Products of exosmosis and root falls accumulated in rhizotron conditions so as in natural localities are consumed by the complex of heterotrophic microorganisms. The large number of experiments shows that under rhizotron conditions the amount of rhizospheric microorganisms is significantly higher than in soils. On the rhizotron ROST-4M a number of experiments on studying the dynamics of root diazotrophs in the wheat rhizoplane was carried out by us together with the laboratory of biological nitrogen (Research Institute for Agricultural Microbiology). Strains of bacteria perspective on the nitrogen fixation characteristics were used for the inoculation. It was established that investigated strains became acclimatized differently on wheat roots under rhizotron conditions. Some strains acclimatized weably and strains colonized the rhizoplane activity and kept the high population density throughout the experiment. The similarity of population curves obtained in rhizotron experiments in which inoculated plants were cultivated in the soil was shown.

Integrated investigations carried out with cereal, vegetable and other crops testify the considerable possibilities of studying the regularities of the microflora interaction with root systems in rhizotron ROST-4M conditions.

USE OF MARKERS RAPD FOR IDENTIFICATION AND DETECTION OF *AZOSPIRILLUM* STRAINS IN A SOIL MICROCOSM

FANCELLI, S., BAZZICALUPO, M., DI SERIO, C., GALLORI, E., MARANGOLO, M. AND FANI, R.
Dipartimento di Biologia Animale e Genetica; Via Romana 19, 50125 Firenze, Italy. E-mail:MARCOBAZZI@DBAG.UNIFI.IT

In the search for a strategy to generate specific probes for bacterial identification, as an alternative to other molecular techniques (such as REA, RFLP, rDNA sequences analysis) we exploited the ability of Random Amplified Polymorphic DNA (RAPD) to generate specific markers for a given genome. In our previous works we tested this strategy with isolated strains of the nitrogen fixing soil bacterium *Azospirillum* demonstrating that RAPD can provide DNA probes with different degree of specificity which can distinguish between species or even between strains belonging to the same species (Fani et al., 1993). We here demonstrated that this technique could be appropriately applied to the analysis of free living bacteria and in particular to directly detect the occurrence of particular strains in an environmental sample without isolation. Three RAPD fragments named α4 (from *A. lipoferum* ATCC29731), α1c (from *A. brasilense* Cdr) and α6 (from *A. amazonense* Y2) were tested for specificity against DNA extracted from pure culture of different *Azospirillum* strains. Only one probe, a4, showed to be highly specific.
In order to test the efficiency of these probes in environmental-like conditions we realised a simple soil microcosm in which single *Azospirillum* strains or a mixture of two or three strains were inoculated. DNA extracted from soil was amplified and the amplification products were dot-blotted and hybridised with probes α4, α1c and α6 separately. The α4 probe confirmed, also for DNA directly extracted from soil, the same high specificity showed against DNA from pure cell cultures, giving strong hybridisation signals only with those samples where *A. lipoferum* ATCC29731 was inoculated alone or in combination with other *Azospirillum* strains. Data obtained indicated that probes generated from RAPD markers are useful tools for bacterial identification in environmental-like conditions; but an accurate choice of the RAPD marker is recommanded.

Fani, R. et al. (1993) Molecular Ecology **2**: 243-250.

NITROGEN FIXATION IN WEST SIBERIA AGROCOENOSIS

GAMZIKOV G.P., GAMZIKOVA O.I., SHOTT P.R.
Altai Research Institute of Arable Farming and Plant Breeding,
Nauchny Gorodok, Barnaul, 656910, Russia.

Quantative parameters of biological nitrogen contribution in total *N* balance of regional agrocoenosis in West Siberia potentially reach 40%, but in fact are 8-10%. Among automorphous soils the maximum expression of rhizospheric *N*-fixation was found is in humic layer of Chernosem and dark gray soils.
Formation of N-fixing associations and their functioning depend on many factors with higher plant characteristics dominating. Wide intergeneric, inter- and intraspecific fluctuation of rhizospheric *N*-fixation is determined experimentally. Screening of specific potential of *Triticum L.* genus allows to observe the evolution of *nis*-character, to evaluate its genetic variability, to extract potential sources of high *N*-fixing activity and to propose ways of creating genotypes to amplify *nis*. Diazotrophic complex can be influenced on by means of agrotechnical measures, among which fertilizers are the most effective. Matching of components of associative complex allows to enhance N-fixation rate in rhizosphere of grain crops by 2.5-4.5 times and plant productivity by 25-50%. Studied strains vary in versatility degree: azospirilla had more frequent positive effect, Bioplant had the lowest effect.
The intimate dependence of *nis* on negative anthropogenic factors was estimated on increasing concentrations of heavy metals in soils (*Cd, Pb,Ni*). Pb was the most toxic for diasotrophes which lead to N-fixation reduction in rhizosphere of barley by 15%, of wheat - by 30-32%. Response of nitrogenase complex on heavy metals present in soil was determined by specific peculiarities of plants. Negative influence of heavy metals can be deminished significantly (up to 50%) by means of optimisation of nitrogen nutrition of plants applying fertilisers or inoculation of cultivated plants with active strains of nitrogen fixers.
Ways to control the activity of rhizospheric *N*-fixing complex functioning are as following: genetic and breeding creation of plant genotypes, ecologically adapted technologies of their cultivation and purposeful change of soil biota content.

AMMONIUM INFLUENCE ON NITROGENASE ACTIVITY OF RHIZOSPHERE NITROGEN FIXING BACTERIA IN PURE AND MIXED CULTURES

GLAGOLEVA O.B., ZLOTNIKOV A.K., UMAROV M.M.
Lomonosov Moscow State University, Department of Soil Biology,
Moscow 119899, Russia

The attempts to use pure cultures of rhizosphere nitrogen-fixing bacteria for intensification of associative nitrogen fixation are not always successful, which has been commonly attributed to inadequate knowledge of the ecology of these bacteria. At the same time a significant stimulating effect of mixed cultures of nitrogen fixers on non-legume plants has been found experimentally (Cacciari et al., 1989; Umarov et al., 1993). Most often artificially mixed bacterial cultures were used. In our opinion utilization of natural nitrogen-fixing consortia is more promising as far as these bacterial communities have a constant composition and produce a stronger and more stable effect on agricultural plants.

In soil the activity of rhizosphere diazotrophs is regulated by a complex of environmental factors. In particular, the application of ammonia fertilizers influences greatly on nitrogen-fixing activity of bacteria in rhizosphere.

Five bacterial mixed cultures actively fixing nitrogen were isolated from rhizosphere soil samples. They are proposed to be natural bacterial consortia. The consortia are mostly binary. *Bacillus azotoformans, B. megaterium, B. fastidiosus, Arthrobacter sp., Acinetobacter sp., Cellulomonas sp., Streptomyces globiformis* were identified among their components.

Nitrogenase activity of both pure and mixed cultures is repressed at 10^{-3}M NH_4^+ in liquid medium when exposed to microaerophilic conditions. The activity increases in 1.2-3 times under aerobic conditions with 10^{-6}M NH_4^+.

The activity of the pure cultures is less suppressed by high ammonium concentrations after 10-minute incubation as compared to the consortia. Mixed cultures show considerably higher nitrogenase activity in comparison with pure cultures after 24-hour incubation with 10^{-3}M - 10^{-1}M NH_4^+.

Calculated ammonium concentration in soil solution (when common doses of ammonium fertilizers 25-75 mg N/kg soil are applied) turned out to suppress nitrogenase activity of nitrogen fixing bacterial consortia.

In general, this phenomenon influences considerably upon total nitrogen balance in agroecosystems.

Cacciari I et al (1989) Plant Soil 115(1), 151-155.
Umarov MM et al (1993) Pochvovedenie 9, 70-75.

DIAZOTROPHS AND THEIR EFFECT ON WHEAT

GOVEDARICA M., MILOSEVIC N., JARAK M., MILOSEV D.
Faculty of Agriculture, Institute of Field and Vegetable Crops, Maksima
Gorkog 30, 21000 Novi Sad, Yugoslavia

According to several reports, total yield increase generally ranges for 10 to 30% under the effect of diazotrophic bacteria inoculation (Bashan, Levanony, 1990).

The objective of the investigation discussed in this paper was to study the effect of different diazotrophs on winter wheat yield. Eight strains of *Azotobacter chroococcum* and one strain of *Azospirillum lipoferum* and *Beijerinckia Derx* were selected for the study (Govedarica et al., 1991). The effect of these diazotrophes was studied on winter wheat variety Novosadska rana. The wheat plants were grown in vegetation pots in a greenhouse until the end of growing season. Before sowing, the seeds were inoculated with the studied diazotrophes. For control variants were used in the study: a control with no mineral N added and controls with 0.2, 0.4 and 0.6 g of NH_4NO_3 per pot. The studied diazotrophs increased plant height, number of nodia, length of spike, number of fertile spikelets, and absolute mass of 1000 seeds. The increase of 1000 seeds mass ranged from 2 to 14% with respect to the controls while the increase of other yield components ranged from 5 to 10%. With respect to the control with no N, diazotrophs increased wheat yield. The inoculation with three strains of *Azotobacter chroococcum* and one strain of *Azospirillum lipoferum* affected the yield increase with respect to the control with NH_4NO_3. Diazotrophs brought to increased number of different systematic and physiologic groups of microorganisms and the increase of dehydrogenase and protease activity in soil.

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ISOLATION AND CHARACTERISTICS OF N_2-FIXERS FROM RHIZOSPHERE OF *ANEUROLEPIDIUM CHINESE* PLANTS IN THE GRASSLAND, INNERMONGOLIA

GUAN XIUQING
Institute of Botany, Academia Sinica 141, Xizhimenwai Street 100044 Beijing, P.R.China

At present, work on associative nitrogen fixation of rice, wheat, maize, sugar cane and grain crops have been reported. Associative nitrogen fixing bacteria exists in rhizospere and root surface and even in the cortex cells of roots of tropical and subtropical herbage. But in China, the study of associative nitrogen fixation of the rhizospere of herbage, especially the cereals in dry areas of northern China have been less studied.

Two strains with higherst nitrogen-fixation rate among the six strains of N_2-fixers, have been isolated from the soil of *Aneurolepidium chinense* rhizophere in the Grassland, Innermongolia. According to the characteristics of morphological and physiological properties of these strains, the strain A-1 was assigned to *Klebsiella oxytoca*, the other one B-18 to *Klebsiella pneumoniae* subsp. *pneumoniae*. The two strains have higherst nitrogenase activity at the lowest pressure of oxygen.

K. pneumoniae is found in almost all areas in China, but *K.p.* subsp. *pneumoniae* is rarely found at the dry area in Innermongolia. We isolated two *K.p. pneumoniae* strains which have not been found yet in China in the rhizosphere of graminaceous pasture. Therefore under the special ecological environment of Innermongolia the nitrogen fixing bacteria are also different from other areas.

NITROGEN-FIXING BACTERIA ASSOCIATED WITH MAIZES NATIVE OF OAXACA

HALLBERG, T.B.
Instituto Tecnológico de Oaxaca
Calzadas Tecnológico y Gral. Wilfrido Massieu s/n
(or Apartado Postal 1461)
Oaxaca de Juárez, Oaxaca, 68030 México

Nitrogen-fixing bacteria have been found associated and probably co-evolved with the roots of one cultivar of maize (*Zea mays* L.) native of Oaxaca. By petition of this author, in 1990 the existence of the first of these N_2-fixing bacteria was confirmed in laboratory analysis by Dr. G.J. Bethlenfalvay of the Agricultural Research Service of the United States Department of Agriculture (G.J. Bethlenfalvay, Personal communication) and, in 1992-93 a total of 63 different N_2-fixing bacteria by Dr. R. Ferrera-Cerrato of the Postgraduate College of Mexico who, in the same samples, also found many P-solubilizing and antibiotic bacteria (R. Ferrera-Cerrato, Personal communication). Observations by this author of similar phenomena in many of the thousands of other native maize cultivars in the multifarious agro-ecological environments of this area of origin of maize perhaps 10,000 years ago suggest that a multitude of other beneficial bacteria are to be found. Furthermore, history teaches us that, many centuries prior to the initiation of the European Conquest, the Mexican people had already invented and were using the most accurate calendar in the world and highly-productive, adapted, useful and diversified maize varieties in its principal types - dent, flint, floury, pop and sweet - of worldwide value. Yet, today their descendants are systematically downtrodden, their maize seed discriminated against and their talent for research stifled. The world is now seeking these native values for sustainable agriculture. By mobilization of financial, human and physical-plant resources, a new RESEARCH CENTER ON MAIZES NATIVE OF OAXACA is being established to prepare local native talent as professionals to identify and manipulate these beneficial microorganisms and their ecosystems for sustainable agriculture everywhere. Delayed by the unexpected economic crisis, this Center seeks and will welcome collaboration from financial sources and research individuals and institutions from across the world to help study, utilize and improve native traditional technology, seed and associated beneficial microorganisms, and train personnel, thus propitiating for the native peoples of Oaxaca (stewards of this biological goldmine of inherited germplasm) direly-needed prestige and a decent standard of living.

GROWTH RESPONSE OF WHEAT AND MEDIC TO ROTATION. EFFECT OF PHOSPHORUS.

ISMAILI, M. AND EL ABBADI, K.
Department of Biology, Faculte des Sciences B.P. 4010
BeniMhamed, Meknes, Morocco

Crop rotation including legumes reduces large inputs of inorganic N fertilizer to crops. However, P is a limiting nutrient in many soils in Morocco and the success of rotation of annual legumes and wheat depends on P available in soils. A greenhouse experiment was conducted using annual medic (*Medicago truncatula* cv.Jemalong) and wheat (*Triticum durum* Karim) seeded in 20 L pots in continuous and rotated stands, during five successive crops. Four P rates; 21.8, 43.6, and 65,4 kg P/ha were used. The soil was labelled, also wheat material enriched with 2.08% ^{15}N atom excess was applied at the beginning of the experiment. Medic and wheat shoot dry weights and total N were significantly improved by P treatments. Crop rotation compared with continuous crop was shown to increase the crop N uptake and dry matter production by both species. The addition of P improved the effect of rotation on medic and wheat. Differences in N uptake by wheat following medic and that of continuous wheat, indicated that the residual effects of the legume crop amounted to a maximum of 0.17g N/pot in the second crop. Soil available N increased by medic was due to N added in medic residues left in the soil, and not to a less efficient use of soil mineral N by the medic.

Legumes increased the growth of the succeeding wheat crop, and allowed greater availability of soil mineral N derived from the release of symbiotically fixed N from the breakdown of the roots and nodules. Wheat and medic are compatible in rotation. The wheat improved soil conditions for the following medic. Phosphorus is important to the rotation system as it increases mineralization of N and improved the two components of the rotation.

INFLUENCE OF WEEDS ON NUMBER OF FREE NITROGEN FIXATING BACTERIA IN CORN (*Zea mays* L.)

JAIME, M.A. (1), ULLA, E.L. (2) AND PISCITELLI, F.R (3)
(1), (2) - Microbiologia Agricola.
(3) - Botanica General. Facultad de Agronomia y Zootecnia. Universidad Nacional de Tucuman. CC 125 4000 S. M. de Tucuman. Argentina.

The undergrowth of weeds competes with the cultivation for the space, light, nutrition, water and carbon dioxide at a particular place and time (Kogan, 1992). It have been made studies about the action of some herbicides on nitrogen-fixing microorganisms (Omar et al, 1992); it is also known that some microorganisms produce toxins of herbicide activity, but there are no data about competition of the weeds for this kind of microorganisms, being this the aim of the present work.

The trial was carried out in the experimental field of the Agronomy Faculty, about the cultivation of corn (*Zea mays* L.) cv. Leales 25 R with the following treatments: ground with weeds: inoculated corn with *Azospirillum* spp. and not inoculated corn; ground without weeds: inoculated corn with *Azospirillum* spp. and not inoculated corn. The identified weeds were: *Oxalis refracta* Saint-Hilaire, *Galinzoga parviflora* Cavanilles, *Apium leptophyllum* (Pers.).F.Muell, *Cyperus rotundus* L., *Sorghum halepense* (L.) Persoon (Cabrera, 1968). Bacteria were grown aseptically in NFb medium (Baldani, Dobereiner, 1979) dilutions of soak of corn roots and for the recounting it was used Fisher and Yates table (Vincent, 1975).

The preliminary results indicate that in the plot covered with weeds the number of microorganisms is lesser than in the plot not covered with weeds. Therefore we can conclude that there exist a competition of weeds for the number of free nitrogen- fixating bacteria.

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NITROGEN FIXING BACTERIAL INOCULANTS: PRODUCTION PROCESS AND APPLICATION FOR PLANT PRODUCTION

KHOTYANOVITCH, A.V.
Research Institute for Agricultural Microbiology, Podbelsky
Shosse 3, St.Petersburg-Pushkin, 8, 189620, Russia

Laboratory of Technical Microbiology has developed a process for production of biopreparations containing *Rhizobium, Flavobacterium, Agrobacterium, Arthrobacter, Klebsiella, Enterobacter, Azospirillum, Bacillus* (endophyte). These inoculants are designed to increase productivity of legumes, grain and vegetable crops. The effectivity of application depends upon the properties of soil (especially organic matter content), plant cultivar and geographic locality (in the South of Russia the bioprepatations are more effective).

Almost all these inoculants are produced employing the method of solid state cultivation with the use of the carrier - gamma sterilized peat (radiation dose is no less 2.5 mRad). The suitability of the peat is determinated by a biological method. Inoculation of the sterilized powder-like peat in polyethilene bags is done aseptically. The bacterial strains are grown in liquid nutrient media (a special medium for each strain) using available and cheap ingredients. Usually we obtain titers no less than 5-20 billion cell/ml. The Russian standard for bacterial cell content in a ready peat biopreparation is: *Rhizobium* - no less than 2.5 billion cell/g, associative nitrogen fixing bacteria - no less than 6 billion cell/g. The shelf life of inoculants is 6 months.

A standard amount of the inoculants per a hectare dose of seeds is 0.6-1 kg. For potato tubers inoculation - 3 kg are recommended per a hectare dose. For some strains of *Rhizobium* and *Agrobacterium* a method for production gel forms of the inoculants have been developed. For this the strains producing large amounts of exopolysaccharides during growth on special media are used. Gel-inoculants are more effective than those which do not produce exopolysaccharides.

Successful production and application of the inoculants in plant production is due to:
1) availability of an active and technological strain (technologicity means ability to produce a high quality inoculant), 2) development of a simple and suitable process for biopreparation production, 3) optimal locality (part of the plant to be treated) for application of the biopreparations, plant cultivar, soil, climate.

Annual amount of the inoculant production in FSU was about 4 mln. hectare portions. All these biopreparations are designed for seed inoculation. However, this way is not optimal for biological reasons. We are at present developing a liquid form of the biopreparations to be applied outside the roots (living bacteria or their metabolites?).

PRODUCTION OF THE STABLE BIOPREPARATION OF AZOSPIRILLUM

KHOTYANOVICH A.V., LEBEDEVA E.I.
All-Russia Research Institute for Agricultural Microbiology,
Podbelsky Shosse 3, St.Petersburg-Pushkin, 189620, RUSSIA

Nitrogen-fixing bacteria from genus Azospirillum can be classified as "non-technological" cultures and it is a complex problem to manufacture biopreparation on its base with the high and stable number of bacteria. In the liquid medium the vegetative cells inhibited by itself and died, especially at high number of bacteria 5-8 10^9 CFU/ml. In the diluted suspension cells are more viable however dilution decrease the quality of biopreparation.

Study of the physiology and technology of different Azospirillum strains (mainly A. lipoferum) should be able to work out the simple procedure for cell maintenance in the cyst form that can ensure the high number of bacteria in the liquid, geleous and peat based preparation during the 6 months storage. The application of the preparation made by this procedure gives the positive results in agriculture. From the other hand it seems difficult to achieve the high percentage of cysts recovery even in laboratory.

EFFECT OF INOCULATION WITH NITROGEN FIXING BACTERIA ON HEAVY METALS AND RADIONUCLIDES UPTAKE BY THE PLANTS GROWN IN CONTAMINATED SOILS

KOZHEMYAKOV A.P. (1), CHERNYAEVA I.I. (1), BELIMOV A.A. (1), KUNAKOVA A.M. (1), FOMENKO E.V. (1), YUDKIN L.Y. (2), STEPANOK V.V. (2), RABINOVITCH G.Y. (2), CHERNENOK I.V. (3)
(1) Research Institute for Agricultural Microbiology, Podbelsky Sh.3, Pushkin-8, Saint-Petersburg, 189620, Russia.
(2) All-Russia Research Institute for Agricultural Use of Land-Improved Soils, P.O. Emmaus, 171330, Tver, Russia.
(3) Inst. Radiation Physico-Chemical Problems,Minsk, 220109, Byelorussia.

Under field conditions inoculation of barley with biopreparations of associative bacteria (Azorizin, Agrofil, Mizorin, Flavobacterin) decreased significantly the percentage and accumulation of lead in grain when the plants were grown in artificially contaminated soil. It was found that inoculation changed a balance of various elements (P, K, Ca, Zn, Co, Br, Ba, Sr, Ce, Ni, As) in barley plants. Contamination of soil with lead had no negative effect on survival of the introduced bacteria on roots. In a pot experiment *Az.chroococcum* alone or mixed with *Rh.meliloti* significantly increased the biomass and decreased the percentage of cadmium in alfalfa plants when the soil was strongly pollued with cadmium. The biopreparations of associative and nodule bacteria were used in field experiments with wheat, barley, carrot, beetroot and clover grown in Byelorussian regions which have been exposed to radio-active contamination. It was found that the content of radionuclides (^{137}Cs, ^{40}K, ^{90}Sr) in the inoculated plants was significantly lower than in uninoculated controls. The ability of the studied strains to inhibit accumulation of caesium by the inoculated plants has been described using the radio-active isotope ^{134}Cs (Dritchko et al., 1991). Our results demonstrate that application of biopreparations can decrease the uptake of heavy metals and radionuclides by the plants as well as minimize unfavorable effects of toxic metals on the plants. It is possible that the introduced bacteria change availability and toxicity of the elements for the plants due to dissolution or immobilization of metals and radionuclides: oxidation- reduction reactions which effect valence state and solubility of elemets; changes in pH and Eh; production of sequestering agents; bioaccumulation and biosorption (Francis, 1994). Our results showed that nitrogen-fixing bacteria exhibit a high enzymatic reduction activity. On the other hand, bacteria can improve adaptation of the inoculated plants to unfavorable environmental conditions due to stimulation of plant growth as well as enhancement of mineral and water nutrition (Bashan, Levanony, 1990; Belimov et al., 1994).

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ESTIMATION OF NITROGEN FIXATION BY ASSOCIATIVE RHIZOBACTERIA USING TRACER TECHNIQUE

KOZHEMYAKOV A.P., TCHEBOTAR V.K., BELIMOV A.A.
Research Institute for Agricultural Microbiology,
Podbelsky Shosse 3, St.Petersburg-Pushkin 8, 189620, Russia

Nitrogen fixation from air is one of the main modes of action of associative rhizobacteria on the non-leguminous plants. But amount of fixed nitrogen depend on the environmental conditions and plant genotype. Application of ^{15}N labelled fertilizers helps to estimate nitrogen balance of non-leguminous plants when they have been grown on the pure sand or soil. Experiments with the usage of tracer technique showed that inoculation of barley and sorghum by nitrogen-fixing *Azospirillum* has strong effect on the nitrogen balance of these plants. Inoculation by *Azospirillum* due to increase of acetylene reduction activity on the barley roots by 5-20 times.Fertilizer usage efficiency (FUE) by inoculated barley and sorghum plants has been increased by 15-30%. This is very important for the enhanced nitrogen uptake by non-leguminous plants. Calculation of nitrogen balance of barley and sorghum when they have been grown on the sand (modelling system) showed that nitrogen fixed from air consist up to 20-30% of total nitrogen in the plant. But up to 70-80% of total nitrogen fixed from air has been accumulated in the substrate, thus enrich it. The part of nitrogen fixed from air in the inoculated plants is: - 30-40% in the roots - 10-20% in the shoots

Increasing the dose of fertilizer lead to increasing part of nitrogen fixed from air in the non-leguminous plants. Inoculation by *Azospirillum* increased the productivity of nitrogen fixation in the rhizosphere of barley and sorghum by 5-6 times.
Application of associative nitrogen fixing rhizobacteria has a great effect on the nitrogen balance of barley and sorghum increasing FUE and activity of nitrogen fixation in the plant rhizosphere.

NH_4^+-EXCRETING *ENTEROBACTER GERGOVIAE 57-7* MUTANTS AND ITS NITROGEN SUPPLY TO MAIZE HOST

LI J-D, LI Y-X, DONG Y-M, WANG J-W and AN Q-L
Institute of Botany, Academia Sinica, Beijing 100044, China

Enterobacter gergoviae 57-7 was an associative diazotroph isolated from maize roots. Methylamine-resistant mutants of *E. gergoviae 57-7* by mutagenesis with a transposable element Tn5 were selected. The mutant MG61 excreting 2mmol/L ammonium during diazotrophic growth was studied in detail. The mutants MG61 grew more slowly than wild type did under oxygen-limiting condition or aerobically. The mutants MG61 grew poorly in the medium containing 5mmol/L or 30 mmol/L ammonium that means they couldn't ultilize ammonium for a nitrogen source but it expressed up to 86% of the fully depressed nitrogenase activity when grown in the medium containing 20mmol/L ammonium. The mutants MG61 could utilize glutamate as a sole nitrogen source. In the presence of nitrate (10mmol/L) in the medium, MG61 grew slowly but excreted 7.8mmol/L ammonium(Li Y-X, 1995). As wild type *E.gergoviae 57-7*, mutants MG61 became established in the rhizospheres of in the field grown maize plants at levels of $\sim 10^7$ cells per g of fresh root. In the pot experiments in the maize inoculated with strains, mutants MG61 were responsible for significant increases in root and shoot dry matter(about 12–30%), compared with wild type treated maize plants or with noninoculated controls. Total plant nitrogen accumulation increased as well. By using ^{15}N dilution technique, experiments showed that mutants MG61 incorporated significantly higher amounts of ^{15}N inside root and shoot material than the wild type did. (10% total N derived from nitrogen mutants fixed). Studies indicated that NH_4^+-excreting mutants MG61 support the nitrogen supply of the host plants.

Li Y-X et al(1995)Acta Botanica Sinica.11:

THE EFFECTIVENESS OF PREPARATIONS OF ASSOCIATIVE NITROGEN-FIXING BACTERIA ON POTATO.

LUKIN S.M.,SIMAKOV G.V.,MOROSOV A.M.
All-Russian Research Design and Technological Institute of
Organic Fertilizers and Peat, s. Vjatkino Vladimir region,
601242,Russia.

The effectiveness of nitrogen-fixing bacteria preparations obtained in Research Institute for Agricultural Microbiology (St.Petersburg- Pushkin) has been studied in the field experiments in soddy podzolic loamy soil during 1991-1993. The investigations were carried out with the following preparations: Extrasol N15, KO (titre of bacteria of genus *Pseudomonas* accordingly $12.15x10^9$ and $22x10^9$ g^{-1}), *Azotobacter* N12 ($3x10^9$ g^{-1}), *Serattia* N218 ($9.24x10^9$ g^{-1}).The rates of application of the above-mentioned preparations were 0.95, 1.90 and 5.00 kg ha^{-1} in combination with peat - manure compost in dose 30 t ha-1 locally in the root zone of potato "Anosta".

The application of bacterial actual materials increased the potatoes yield by 18-36 c ha^{-1} or 12-25%. The maximal microbial carrying capacity resulted in the highest yield of potato for all preparations (except Extrasol N15). The application of peat-manure compost 30 t ha^{-1} in combination with Extrasol KO and *Serattia* N218 in dose 5.00 kg ha^{-1} increased the potatoes yield by 55 c ha^{-1} or 43%.

The comparison of bacterial fertilizers extra yields with nitrogenous fertilizers extra yields shows that the application of 5.00 kg ha^{-1} Extrasol KO and *Serattia* N218 is identical with the placement of 36 kg ha^{-1} nitrogen. Extrasol N15 - 25 kg ha^{-1} and *Azotobacter* N12 - 22 kg ha^{-1} had less nitrogen-fixing capacity.

The mechanism and application technology of bacterial preparations in combination with organic fertilizers have been worked out for potato. The investigations were carried out according to "Interbionitrogen -2000" programm.

ASSOCIATIONS OF NITROGEN-FIXING BACTERIA WITH WINTER RYE

MALTSEVA N.N., NADKERNICHNAYA E.V., KANIVETS N.A.
Institute for Agricultural Microbiology UAAS, Chernigov,
250027, Ukraine

We demonstrated that winter rye possess a high level of associative nitrogen-fixing activity. The favourable conditions for the development of nitrogen-fixing bacteria of different systematic, ecologic and trophic groups are founded in the winter rye rhizosphere.

More then 250 pure cultures of nitrogen-fixing bacteria and bacterial associations were isolated out of rye rhizosphere on different media. The active nitrogen fixers were detected among the representatives of the genera *Azospirillum, Azotobacter, Bacillus, Clostridium, Pseudomonas*. The active bacteria strains increased nitrogen-fixing activity and productivity of this culture when introduced in rye rhizosphere at pot and field tests by 12-18%. The direct proof of nitrogen-fixing activity in bacteria which belong to the genera *Azospirillum, Azotobacter, Bacillus,Pseudomonas* when interaction with plants was obtained by using ^{15}N isotope.

It was determined that the biological nitrogen entering into the overground plant mass and roots increased under inoculations by 10-14% and 14.8-25.5% respectively. The mutants resistant to the high streptomycin doses (1 mg/ml) were obtained for studying azospirills and pseudomonads striking in rhizosphere. In pot and field tests were shown that introducing mutants develop in winter rye rhizosphere, where their numbers are 30-50 thousand on 1 g of roots at the end of the plant vegetation. Thus it was determined that nitrogen-fixing bacteria form associations with plants in winter rye rhizosphere.The active strains of nitrogen-fixing bacteria were selected for using them as a winter rye inoculants.

CONTRIBUTION OF NODULATED LEGUMES ON THE GROWTH OF *ZEA MAYS* L. UNDER VARIOUS CROPPING SYSTEMS.

MANDIMBA, G.R.
Laboratoire de Biotechnologie. Institut de Développement Rural I.D.R.
Km 17, Université, B.P.13346. Brazzaville, Congo.

Mucuna pruriens, Crotalaria juncea, Cajanus cajan and *Arachis hypogaea* are well nodulated by indigenous rhizobia of soil in the Congo. Nodule numbers per plant range from 8 to 148, nodule mass from 88 to 305 mg per plant and coloured nodule is greater than 72%. Recent studies reveal that the contribution of *A. hypogaea* on the growth of *Zea mays* L. in intercropping system is equivalent to an application of 100 kg N fertilizer per ha at a ratio of plant population densities of one maize plant: four groundnut ones. In addition, crop N yield is higher in *M. pruriens* (340 kg/ha) compared to *C. cajan* (114 kg/ha), followed by *C. juncea* (72 kg/ha) and *A.hypogaea* (39 kg/ha). The values of contribution of legume species as green manure on the grain yield in maize crop are equivalent to an application of N fertilizer of 97 kg N/ha, 89 kg N/ha, 78 kg N/ha for *M. pruriens*, both *C. cajan* and *C. juncea*, and for *A. hypogaea* respectively. There is an evidence on contribution of nodulated legumes to the growth of maize crop.

EFFECT OF INOCULATION WITH DIFFERENT STRAINS OF *AZOTOBACTER CHROOCOCCUM* AND *AZOSPIRILLUM LIPOFERUM* ON SOME VEGETABLES

MILOSEVIC N., GOVEDARICA M., JARAK M.
Faculty of Agriculture, Institute of Field and Vegetable Crops,
Maksima Gorkog 30, 21000 Novi Sad, Yugoslavia

Plant inoculation with diazotrophic bacteria can bring to significant changes of various plant growth parameters (Bashan, Levanony, 1990; Govedarica et al., 1993).
Sweet pepper and tomato seeds were inoculated with five strains of *Azotobacter chroococcum*. Carrot seed was inoculated with two strains of *Azospirillum lipoferum*. The seeds were inoculated with 10^8 cfu/ml. The plants were grown on sterilized sand with non-nitrogen nutrient solution for 30 days. Each vegetable was represented by two varieties.
Plant inoculation with diazotrophic bacteria can significantly affect the length, mass and content of N in root and above-ground plant part.
Effect of inoculation on the studied parameters was different and depended on bacteria strain, plant and variety. Three *Azotobacter* strains had positive effect on the length, mass and N-content, one strain had no effect, while one strain had negative effect. On average, the strains affected the increase of the above-ground part (18-36%) and the root (6-22%) of sweet pepper. Considering tomato, the strains affected the increase of N-content in the above-ground part from 10 to 49% and in the root from 8 to 31%. Both strains of *Azospirillum lipoferum* had positive effect on the parameters studied in the above-ground part and root of carrot.

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ASSOCIATIVE NITROGEN FIXATION IN SUGAR BEET

MRKOVACKI, N., MEZEI, S., POPOVIC, M.[1], KOVACEV L., SARIC, Z. AND DJUKIC, D[2]
Institute of Field and Vegetable Crops, 21000 Novi Sad, Maksima Gorkog 30, Yugoslavia
1. PMF, 21000 Novi Sad, Trg Dositeja Obradovića 3, Yugoslavia
2. Faculty of Agronomy, 32000 Čačak, Cara Dušana 34, Yugoslavia

To be successful inoculants bacteria applied to seeds or seedlings should colonize the rhizosphere with a population density and for a length of time sufficient to stimulate growth and yield of inoculated crops (Jagnow, 1987).

Using isolates of *Azotobacter chroococcum* strains (1, 2, 3, 5, 8, 9, 11, 12, 14, 15) from the rhizosphere of sugar beet grown on a chernozem soil in the Vojvodina Province, we studied physiological characteristics of nitrogen fixation in pure culture and in the association with seedlings of two sugar beet genotypes (Ns-Hy 11 and Dana).

Nitrogenase activity in pure culture showed that the strains are different with respect to their activity (21-79 nmol C_2H_4/ml suspension h^{-1}).

Associations were made in vitro between calli of two sugar beet hybrids and *Azotobacter* strains. As in the previous experiments, the callus mass increased in the association with *Azotobacter* from 500 to 1500 mg which depended on *Azotobacter* strain (Mezei et al., 1993).

In the experiment with three nitrogen levels (1/8 N, 1/4 N, 1 N) and without N in the medium, nitrogenase activity was detected only in the medium without nitrogen and with 1/8 N. Depending on the strain, the activity of nitrogenase varied from 11.29 to 30.98 nmol C_2H_4/plant/h in the associations with the hybrid NS Hy 11 and from 13.07 to 42.64 with Dana. Both hybrids are "nis" positive (N fixation supporting) which was proved in our previous studies (Mrkovački et al., 1995).

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AMMONIA EXCRETION BY *AZOSPIRILLUM* AND *HERBASPIRILLUM* MUTANTS RESISTANT TO ETHYLENE DIAMINE

NIRMALA, C.B. PALANIAPPAN, S. P. AND THOMAS, J.
Centre for Biotechnology, SPIC Science Foundation, 110,
Mount Road, Guindy, Madras 600 032 India.

Azospirillum in contrast to *Rhizobium* does not release fixed nitrogen to its environment. Ethylene diamine (ED), an analogue of ammonia has been used to select mutant cyanobacteria which excrete high amounts of ammonia into the medium. Strains of *Azospirillum* and *Herbaspirillum* resistant to ED, a competitive inhibitor of glutamine synthetase were selected and screened for the ammonia excreting ability using Nessler's reagent (Krug et al., 1979).

Spontaneous mutants of *Azospirillum* and *Herbaspirillum* strains resistant to 0.05% ED, occured at a frequency of 3×10^{-8} and 5×10^{-7} per cell. Of thirty colonies tested, two mutants, one each of *Azospirillum* and *Herbaspirillum* excreted substantial amount of ammonia in nitrogen free semisolid medium. In a laboratory study, the dry matter production of rice plants inoculated with mutant strains was significantly greater than those inoculated with wild types. However, ther was no difference in shoot length and root length. The performance of the mutant, SL ED5 was tested in rice and cotton fields. The dry weight of plants treated with both wild and mutant strains was significantly greater than that of uninoculated cotton plants. Since, the plants in our experiment were not provided with any other nitrogen source, the availability of mineral nitrogen was limiting factor for plant growth. As the number of rhizosphere colonizing bacteria was similar in both wild and mutant strains, this effect may be attributed rather to an additional supply of nitrogen than of bacterially produced plant growth substances. Pedrosa and Yates (1984) described ED resistant *A. brasilense* strains that released excess ammonia and Christiansen-Weniger and Van Veen (1991) reported the behaviour of *A. brasilense* ED mutants in rhizosphere of wheat, grown under controlled conditions. Our experiments suggest the potential of ammonia-excreting mutants for use in rice and cotton crops.

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BIOLOGICAL DIVERSITY AND COMPETITIVENES OF NITROGEN-FIXING BACTERIA *ERWINIA CAROTOVORA*

PISHCHIK, V.N.(1), CHERNYAEVA, I.I.(1), LUKYANENKO, E.O.(1), LEBSKY, V.K.(1), VOROBYOV N.I.(1), LAZAREV, A.M.(2), KOZLOV, L.P.(2)
(1) All - Russia Research Institute for Agricultural Microbiology,
(2) All - Russia Research Institute of Plant Protection, St.Petersburg - Pushkin, 189620, Russia.

75 representative strains of *Erwinia carotovora* (Lazarev, 1985) were compared with the bacteria belonging to the family *Enterobacteriaceae* using numerical taxonomic procedures (Sokal, Michener, 1958). The avirulent bacteria differed from virulent *Erwinia carotovora* in a number of properties: lack of pectatliaze isozymes production, the activity of sugars, alcohol and glycoside fermentation rates, activity of nitrogen fixing complex, the resistance to sodium chloride and nitrate and also to antibiotics, reaction in litmus of milk, utilization of inositol and gelatin liquefaction. The study of bacterial ultrastructure and conditions of rhizosphere colonization was made both in the H- 300 transmission electron microscope and in the JSM - T200 scanning electron microscope (Lebsky et al, 1981). We could not find differences between the peculiarities of colonization of tomato rhizosphere with avirulent or virulent *Erwinia.* The root cap was devoid of bacteria. Like the root cap, the extension zone was also relatively bacteria free. The absorbing and conducting zones were colonized by numerous bacteria. The microorganisms were not monotonously distributed on the root surface, most of them occurred in discrete colonies. The bacteria colonized the destroyed rhizodermal cells. A zone with a great quantity of fibrils was formed only around the virulent bacteria in the mucilage. Such zone was absent around avirulent *Erwinia carotovora.* Thus the avirulent *Erwinia carotovora* competed for energy - rich sites with virulent bacteria. In normal situation the avirulent *Erwinia carotovora* prevailed over the virulent strains in the microbocenosis when plant nitrogen nutrition was balanced. The virulent bacteria dominated over avirulent strains under unfavorable nitrogen nutrient conditions for the plants. The content of nitrates in the potato tubers increased with increasing the nitrogen fertilizer doze. The virulent bacteria dominated over the avirulent ones in the microbocenosis. The consequences result in an increased rotting of tubers which are badly preserved. It was established that this unfavorable for the plants situation may be improved through the inoculation with avirulent strains of *Erwinia carotovora* having a high competitiveness.

Lazarev AM (1985) Abstract of dissertation, (In Russian) Leningrad, 18 P.
Lebsky VK, et al (1984) Bull. RIAM (In Russian), 33, 64-66.
Sokal R, Michener CD (1958) Univ. of Cansas Sc. Bul. 38, p 1409.

PHOTOTROPHIC NONSULFUR PURPLE BACTERIA AS ASSOCIATIVE DIAZOTROPHS

RED'KINA T.V., MITUSHINA L.L., LYSENKO A.M.
Laboratory of Soil Microbiology, Institute of Microbiology RAN,
Moscow, 117811, Russia

It was shown that nonsulfur purple bacteria can occur at the rhizosphere of higher plants (rice,barley,oat and some others). The number of nonsulfur purple bacteria on the roots of rice was as high as 10-70 millions of cells per g roots 2,5 millions for oat and barley and 10-100 thousands for brome grass and fascue grass.Composition of these bacteria depends on plant variety. A binary association nonsul purple bacteria was found in the rhizosphere of some rice varieties . Association of four components was revealed in the other rice varieties.Among those only one component is known species - *Rhodobacter spheroides*,others do not have analogous genera and species. Only one species of *Rhodobacter spheroides* with marked strain differences was found in rhizosphere of oat, barley,fascue grass,brome grass.The isolated strains had abilities to grow and fix nitrogen phototrophically in aerobic and anaerobic conditions in light and heterotrophically in dark. The strains had different ability to grow in aerobic and anaerobic conditions in light.Some strains are denitrificators. Together with nitrogenase activity the isolated strains have another peculiarities which are very important for the plants. They were shown to produce growth - activating substances for plants as well as antibiotics inhibiting the growth of phytopathogenic fungi providing the biological defense of higher plants.

RESPONSE OF RED BEET TO INOCULATION WITH N_2-FIXERS IN DIFFERENT SOILS

SHABAYEV V.P., SMOLIN V.YU., SAFRINA O.S.
Institute of Soil Science and Photosynthesis, Russian Academy of Sciences,
Pushchino, Moscow region, 142292, Russia

The effects of N_2-fixing bacteria of the *Pseudomonas* genus on the growth of red beet (*Beta vulgaris var. rapata* L.) in the soils with different fertility levels have been studied in a 3-year microfield experiments. Bacteria were isolated from oat rhizosphere and had N_2ase activity (8,4 mM C_2H_2 $h^{-1}ml^{-1}$ of medium). The plants were grown in pots (0.33x0.33x0.33m) without bottoms buried in soil and filled with different soils on the background of ^{15}NPK fertilizers. Seedlings were inoculated with 10^7 CFU of bacteria per plant. Survival of bacteria in the rhizosphere and rhizoplane of plants grown in pots containing 1 kg of different soils were monitored using kanamycin- (200 μg ml^{-1}) and streptomycin- (200 μg ml^{-1}) resistant strains. C_2H_2 reduction of initial soil samples were determined after their incubation with glucose (2% dry wt) at 28°C and 60% WHC during 24h. Acetylene reduction was also assayed with intact plants grown in pots containing 4 kg of soil. The effectiveness and number of bacteria in the rhizosphere and rhizoplane depended greatly on their survival and N_2ase activity in the soil. It was found that bacteria best survived and stimulated plant growth in the soil with negligible level of N_2ase activity. In contrast, lesser survival and the absence of the bacterial effect was found in the soil with high level of N_2ase activity. The determination of this parameter makes possible the forecasting of the effects of bacteria on plant growth in different soils. Growth responses of red beet to inoculation with bacteria were accompanied by an increase in leaf area, photosynthetic activity (CO_2 exchange), nitrate reductase activity of leaf (*in vitro*) and nitrate concentration in plants, including roots. Since the photosynthetic activity per unit (dm^2) of leaf area in inoculated plants did not change, the positive effect of bacteria on plant growth and photosynthesis consisted of an increase in leaf area that was probably conditioned by phytohormone production by these microorganisms. Bacteria increased plant weight a result of N_2ase activity stimulation in the rhizosphere and the rise of N uptake in plants, including «biological» nitrogen (determined by ^{15}N-isotope dilution), as well as other macronutrients and micronutrients. The increases of N_2ase activity in the rhizosphere and incorporation of fixed N into red beet inoculated with bacteria occur during the second and third months in the vegetative period. A nitrogen balance experiment, carried out on plant grown in pots containing 30 kg valley soil (Oka river) constitute evidence for a significant contribution of plant-associated N_2 fixation in red beet.

NEW METABOLITE WITH FUNGISTATIC ACTIVITY PRODUCED BY ASSOCIATIVE NITROGEN-FIXING BACTERIA BELONGING TO GENUS *FLAVOBACTERIUM*

SHENIN Y.D., KRUGLIKOVA L.F.(1), VASSYUK L.F., KOZHEMYAKOV A.P., POPOVA T.A., TCHEBOTAR V.K. (2).
(1) Research Technological Institute of Antibiotics and Medical Enzymes, Rizhsky Prospect 41, St.Petersburg, Russia.
(2) Research Institute for Agricultural Microbiology, Podbelsky Shosse 3, St.Petersburg-Pushkin 8, 189620, Russia.

Biopreparation Flavobacterin is created on the base of strain L-30 belonging to genus *Flavobacterium* for increasing the productivity of cereal, fodder crops and vegetables. Seed inoculation by flavobacteria increased the yield of cereals by 0.3-0.5 t/ha, fodder grasses by 1.4-1.8 t/ha, fodder sorghum by 2.4-4 t/ha,vegetables by 1.7-6 t/ha. Bacterization has improved the plant quality by increasing the content of protein, phosporus, lyzine and ascorbic acid.

Mode of positive action of biopreparation is connected with active nitrogen fixation on the plant roots as well as with the improving of mineral and water uptake, phytohormones production and fungistatic activity.

Numerous greenhouse and field tests data showed that the flavobacteria produce substances suppressing phytopathogens, causing *Rhizoctonia, Fuzarium* and *Uncinula* affection.

Flavocin has been isolated from the culture broth of *Flavobacterium sp.* strain L-30. Antibiotic exhibit high activity against phytopathogenic bacteria and fungi. It was isolated as amorphous power readily soluble in lower alcohols, chloroform and unsoluble in water, acetone and most other organic solvents.

Flavocin gave positive response to ninhydrin and Drangendorff reagents but was negative to Erlich, anthron reactions. The UV-spectrum showed a maximum at 365-272 nm. In H_1NMR-spectrum were observed chemical shift (CD_3OD) of 0.94 (tr), 1.17(d), 1.34(s), 1.65(m), 2.08(m), 2.38(tr), 3.36(s), 7.20(m), 7.97(m). The IR- spectrum is potassium bromide. The following peaks are evident 1720, 1640, 1390, 1330,1140, 760, 630 cm-1. In the mass-spectra was established ions 605 (M+), 578, 552, 412, 368, 313, 256, 236. Hydrolisis of flavocin by 6n HCl at 110oC for 20 hours afforded treonine, glycine, alanine.

Obtained physic-chemical and biological data helped to compare flavocin with the known antibiotics isolated from bacteria:beta-lacton, beta-lactam, nucleozide, peptide e.a.) Comparision showed that antibiotic flavocin has an original composition.

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NITROGEN-FIXING RHIZOBACTERIA:FUNGISTATIC MODE OF ACTION

TCHEBOTAR V.K.,POPOVA, VASSYUK L.F.,KOZHEMYAKOV A.P.
(1) PUKHAEV R.V., OKAZOVA A.G. (2)
(1) Research Institute for Agricultural Microbiology, Podbelsky Shosse 3, St.Petersburg-Pushkin 8, 189620, Russia
(2) North-Caucasian Research Institute of Mountain and Foothills Agriculture, p/o Michailovskoe, North Osetia-Alania,363110, Russia

The ability of nitrigen-fixing rhizobacteria to reduce the affection of non-leguminous plants by phytopathogens has been shown in pot and field tests carried out in different regions of Russia. Results of the potato growing on the soil infected with *Rhizoctonia solani* during three years showed that inoculation by *Flavobacterium spp.*, strain L-30 decreased plant affection by rhizoctoniosis by 2-3 times.Yield of potato tubers increased by 25-50% and affection by rhizoctoniosis, bacterial rot and common scab of potato decreased by 2-3 times due to inoculation by *Flavobacterium* in the field tests in North-Western region of Russia.Inoculation of potato by the same strain of flavobacteria in the North Caucasus decreased affection of tubers by rhizoctoniosis and common scab of potato by 2-15 times, affection of the potato leaves by phytophtorosis and macrosporiosis by 2-3 times.

Action of *Flavobacterium* was more strong comparing with fungicide Arcerid.

Inoculation by nitrogen-fixing rhizobacteria decreased affection of:

-cereals by powdery mildew and root rot by 2-3 times

-sugar beet by cerosporosis and phomosis by 1.5-2 times

In the plate experiments were shown that mutual cultivation of associative nitrogen-fixers with the phytopathogens inhibited growth of fungi from genera *Fusarium, Rhizoctonia, Alternaria, Phoma* and *Phytophtora*. Fungistatic action of nitrogen-fixing rhizobacteria depend on conditions of cultivation,media composition and conditions of culture preservation.Aeration and frequent culture replating decreased ability of associative rhizobacteria to suppress phytopathogens.There are different modes of associative rhizobacteria action on the phytopathogenic fungi in laboratory conditions. Thus, *Azospirillum* and *Pseudomonas* strains can produce siderophores; *Flavobacterium* and *Arthrobacter* strains can produce antibiotic substances.

Fungistatic effect is one of the major mechanisms of associative rhizobacteria action on the non-leguminous plants.

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NITROGEN TRANSFORMATION IN RHIZOSPHERE OF PLANTS INOCULATED WITH DIAZOTHROPHS

UMAROV M.M., GLAGOLEVA O.B., MATVEEVA N.G.
Lomonosov Moscow State University, Department of Soil Biology,
Moscow 119899, Russia

It is well-known that nitrogen-fixing activity in rhizosphere as well as plants yield increase considerably under inoculation of non-legume plants with active diazotrophic bacteria. From the other hand, many of nitrogen-fixing bacteria carry out denitrification and heterotrophic nitrification processes (Umarov, 1990). According to these facts one can suppose that ultimate results of bacterial inoculation depend on correlation between the activities of these processes in rhizosphere.

The influence of inoculation with active N_2-fixing strains of *Bacillus*, *Pseudomonas* and natural bacterial mixed cultures on nitrogen fixation, denitrification and heterotrophic nitrification in the rhizosphere of non-legume plants (*Brassica napus, Hordeum vulgare, Phleum pratense, Dactylis glomerata, Festuca pratensis*) was investigated.

All collection homogenous in nitrogen fixing activity in axenic conditions differs considerably in the associations with plants in model and field experiments. So it was divided into three groups of cultures:

1) strains with high nitrogenase activity and low denitrifying activity (about 5% from all number of cultures);
2) strains, showing high denitrifying activity and having no ability for nitrogen fixation (7%);
3) strains with middle levels of nitrogen fixation and denitrification (88%).

Possibility to select strains, that would primary stimulate one of these processes if inoculated was shown.

The inoculation with the cultures from the first group leads to the increase of plants yield on 37-40%, with the strains of the second group - on 20-26%, and with the microorganisms from the third group - on 12-23%.

It is obvious that this difference between the groups of bacteria demonstrates an important role of nitrogen fixation in plants' rhizosphere (side by side with another factors) on yield formation.

Umarov MM (1990) In Bouwman AF, ed, Soils and the Greenhouse Effect, pp263-268, John Wiley & Sons Ltd.

TAXONOMIC POSITION AND EFFICIENCY OF *AZOSPIRILLUM* OF VARIOUS ORIGIN

VASSYUK L.F., KHALCHITSKY A.E., POPOVA T.A.,
TCHEBOTAR V.K.
Research Institute for Agricultural Microbiology, Podbelsky Shosse 3,
St.Petersburg-Pushkin 8, 189620,Russia.

For 10 years the bacteria belonging to the genus *Azospirillum* have been isolated from the soil samples of Russia, Bulgaria, Hungary, Slovakia, Germany, Romania, India, Mexico, Argentina, Egypt and from the rhizoplane of plant of various families. Azospirilla were found abundantly in the soils where rice, maize, legume-cereal grasses had been grown as well as in the rhizoplane of cereals, however they were absent in the soils containing high doses of fertilizers and manure.

At present the Institute has accumulated a collection of azospirilla belonging to *A.lipoferum* and *A.brasilense*. The collection comprises around 100 strains. The identification of isolates was done on the basis of phenotypic and genosystematic tests (G+C content of DNA and DNA/DNA hybridization). Apart from the generally recognized tests the ability to utilize certain amino-acids and application of plant extract when determining the nitrogenase activity in vitro were used as differentiating properties. The viability of the introduced azospirilla strains on the roots of six various cereals has been studied. The specifity of the plant response to inoculation with azospirilla in pot experiments on rice, maize, wheat and pea was revealed.

The comparative study of effectiveness of the selected azospirilla strains in field trials on cereal and fodder crops in North-Western region of Russia and Moldavia has been conducted. A higher positive effect was observed on barley maize, sorghum, certain species of cereal grasses.

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EPIPHYTIC NITROGEN-FIXING CYANOBACTERIA IN ARCTIC VEGETATION

VIGSTAD H. (1), MYKLEBUST R. (2), ENDAL A. (1),
and SOLHEIM B. (1).
(1) Institute of Biology and Geology and (2) Institute of Medical Biology,
University of Tromsø, N-9037 Tromsø, Norway.

The main input of biologically fixed nitrogen in a High Arctic ecosystem at Kongsfjorden, Spitsbergen (79°N, 12°E) was found to be due to epiphytic cyanobacteria on different bryophytes. Most of the cyanobacteria were located in the space between the leaves and the stem of the moss. The number of cyanobacteria observed correlates with the portion of the moss plants where the highest rates of nitrogen fixation were found - specially, between 0.5 and 1.0 cm from the apex. The ability to support an epiphytic flora varied greatly between bryophyte species. In particullar, *Calliergon richardsonii* and *Sanionia uncinata* seemed well adapted to harbour epiphytic cyanobacteria.

The extent of nitrogen fixation varied with growing location. The rate of nitrogen fixation was affected by, grazing and bird dropping. In a geese grazing area values were found with a maximum of 693.6±1.5 nmol ethylene h-1g[dry weight]-1 while maximum for ungrazed areas was 65.3±16.6 nmol ethylene h-1g[dry weight]-1. This is due to the removal of nutrients from the environment by the herbivores. In the grazed area in addition to those cyanobacteria found on bryophytes, nitrogen fixing cyanobacteria were found on grass.

Under cliffs harbouring colonies of birds feeding in the sea, biological nitrogen fixation was inhibited by bird droppings.

The high plant productivity, supporting heavy grazing, strongly indicates an effective transfer of fixed nitrogen to the plant community.

THE PECULIARITIES OF FORMING ASSOCIATIONS "GRASSES-DIAZOTROPHS".

VOLKOGON V.V., LEMESHKO S.V., MAMCHUR A.E.,
VOLKOGON E.I.
Institute for Agricultural Microbiology UAAS, Chernigov,
250027, Ukraine

We demonstrated that the composition of diazotroph associations of root zone for some fodder cereals species such as *Lolium perenne, Zerna inermis, Phleum pratense, Phalaroides arundinacea* and their seeds is identical in composition. At an example of common ryegrass by electron microscopic studying of the seeds is shown that bacteria are localized under the seed skin in the inter cells of aleuronic layer and endosperm.The associate nitrogen-fixers were isolated out of the surface sterilized seeds of named plant species. The seeds were inoculated by streptomycin resistant bacteria before sowing them in soil. The streptomycin resistant strains were isolated out of the seeds after new yield.In spite of not high bacteria numbers in seeds (up to 100 cells in one seed) when germinating of the latter the cell numbers significantly increased and achieved 10^5 - 10^6 in 1 g of plant roots. These evidences show the close connection of plantsand microorganisms development and specific associations "plant-diazotrophs" formed at the stage of seeds germination.

"Seed" diazotrophs numbers in plants root zone increased when physiologically optimal doses of mineral nitrogenous and molibdenum fertilizers were applied. More perspective factor for numbers regulation of associative nitrogen fixers in grasses root zone and their activity is the plant treatment with growth - stimulated substances. The latter are the analogues of phytohormones of auxin and cytokinin classes. Their action at the diazotrops numbers and activity was indirect by root system increasing, photosynthesis and plant nitrate reductase activization.All this leads to the increasing of nitrogen-fixers providing them with energy substrate and more active nitrogenase synthesis in conditions of nitrogen compounds deficiency.

MIZORIN AN ASSOCIATIVE NITROGEN-FIXING BACTERIA-BASED PRODUCT TO CONTROL SCLEROTINIA IN SUNFLOWER

YAKUTKIN V.I.(1), VASSYUK L.F.,KOZHEMYAKOV A.P.(2)
(1) All-Russian Plant Protection Institute (VIZR),Podbelsky
Shosse 3,St.Petersburg-Pushkin 8,189620,Russia
(2) Research Institute for Agricultural Microbiology, Podbelsky
Shosse 3,St.Petersburg-Pushkin 8,189620,Russia

Mizorin,a product based on strain of associative nitrogen-fixing bacteria Arthrobacter mysorens when tested in the field in different soil and climate areas showed good effects on yield of sorghum,maize,sugar and fodder beets,potatoes,topinambur,vegetable marrows,radish and sunflower.

Its combination with rhizobia resulted in increased productivity of lupin and peas.

As compared to other test products, Mizorin significantly decreased the damage caused by Sclerotinia sclerotiorum to sunflower in field tests.Thus for example on mid-deep chernozem soils in the Voronezh area the percent of damage of Yenisei cultivar was 0.5-+0.5% in the Mizorin treated plants against 20.5+-1.6% in the control.Its biological efficiency in controlling the desease reached 97.6%. The product was as effective against Sclerotinia as 8-year crop rotation where the damage caused did not exceed 2.0+-0.8%.

Thus the strain of Arthrobacter mysorens does not only possess the nitrogen- fixing ability but also can inhibit the causative agent of Sclerotinia in sunflower.

INTEGRATED FERTILIZATION MANAGEMENT OF RICE WITH CYANOBACTERIZATION, NITROGEN, TRACE ELEMENTS AND FUNGICIDE FOR ENHANCING CROP PERFORMANCE AND INCIDENCE OF NATURAL INFECTION WITH SOME FUNGAL PESTS IN THE NILE DELTA

YANNI, Y.G. (1), SEHLY, M.R. (2)
(1) Cyanobacteria, Azolla and Azospirilla Research Section, Sakha Agricultural Research Station, Kafr El-Sheikh 33717, Egypt.
(2) Plant Pathology Research Section, Rice Research and Training Center, Sakha, Kafr El-Sheikh 33717, Egypt.

Inoculation of Egypt rice fields with cyanobacteria increased yield by about 30% with saving of 1/3 to 1/2 of the recommended rates of fertilizer-N. Asymbiotic dinitrogen fixation in the rice ecosystem is not the only contribution. Prevention of growth of aquatic weeds, increasing P-availability, decreasing sulphide injury, aiding soil particle aggregation and incidence of rice infection with the blast fungus *Pyricularia oryzae* and infestation with stem borers and leaf miners were also indexed.

A fiels of the rice variety Giza-176 was inoculated with a soil-based inoculum containing the cyanobacteria: *Anabaena cylindrica, Anabaena oryzae, Aulosira fertilissima, Nostoc muscorum* and *Tolypothrix tenuis*, applied at 100 kg fresh material (90% mosture)/ha 5 days after transplanting, along with urea at 72 or 144 kg N/ha in two equal doses: 25 days after transplanting and at midtillering. Each of the macro plus micro-elements compound "Vitafort" (N, P, K, Mg, Fe, Mn, Zn, Cu, B, Mo, Co, Vitamin B, meta-indole-acetic acid, aminoacids and growth promotion substances), the fungicide Sportake-45 and a combination of 50:50 of the two compounds were applied as soon as the appearance of fungal infection symptoms (during the late panicle initiation stage).

Cyanobacterization and 72 kg N/ha along with 50/50 Vitafort plus Sportake-45 induced the maximum rice growth and performance as indexed by the plant height, productive tillering, grain and straw yields and N-contents, 1000-grain weight, harvest index (% of grain/grain + straw yields) and the agronomic fertilizer N-use efficiency (kg grain yield/kg fertilizer-N) as compared with the 144 kg N received counterparts. Natural infection with *Pyricularia oryzae, Helminthosporium oryzae, Alternaria* sp. and *Sclerotium oryzae*, the causative agents of the rice blast, brown spot, leaf spot and stem rot diseases, respectively, was less in the inoculated sub-subplots comparing with their non-inoculated counterparts, and in case of application of 72 rather than 144 kg N/ha. A mechanism involving a sort of regulation of available-N in the rice ecosystem by the dinitrogen-fixing cyanobacteria is suggested to diminish excessive N absorption and, consequently, decrease plant susceptibility to infection with the fungal pests.

EFFECT OF THE PREPARATIONS OF MICROORGANISMS ON THE SPRING WHEAT YIELD AND QUALITY

ZAVALIN A.A.,CHERNOVA L.S.,KANDAUROVA T.M.
Laboratory of Crop Physiology, Research Institute for Fertilizers and
Soil Science, Moskow, 127550, Russia

The role of preparations of the nitrogen-fixing microorganisms (Rizoagrin and Extrasol) at spring wheat nutrition was studied on a sod- podzolic medium loam soil in central Russia.Seed treatment with microbiological preparations before sowing favoured the significant increase in spring wheat seed production. Grain yield was higher with sufficient rainfall in the first part of the vegetation period, which is due to the better conditions for microorganism development. Wheat productivity was, as rule, the same as that obtained by the application of mineral nitrogen at the rate of 45 kg/ha. Seed treatment with biological preparations together with nitrogen application at the rate of 45 kg/ha produced the same effect as the separate use of the factors mentioned. Biological and mineral nitrogen fertilizers were of equal value to the formation of the grain part in the biological yield. The economic coefficient was, as a rule, similar and made up 0.36 to 0.40 in 1993 and 0.42 to 0.50 in 1994. The protein content in the wheat grain was not influenced greatly by the factors studied. The grain stored up 11 to 12% of protein in 1993 and 10 to 11% in 1994, all the preparations and fertilizers having been taken into account. The use of the nitrogen stable isotope 15N has revealed the role of soil nitrogen, ammonium nitrate nitrogen and fixed nitrogen in wheat yield formation. The main contribution is made by the soil nitrogen while the fertilizers increase the yield from 5 to 20% depending on the year. The plant needs are met by the fixed nitrogen from the air by 24 to 38% in a good year and by 15% in a bad one. When fertilizer nitrogen and microbiological preparations were used together the part of the atmospheric nitrogen decreased while that of the fertilizer nitrogen increased.

SECTION 9

ROUND TABLE DISCUSSIONS ON URGENT ISSUES

NITROGENASE CATALYSIS

LIKHTENSTEIN[1] G.I., THORNELEY[2] R.N.F. *(Conveners).*
[1]Department of Chemistry, Ben-Gurion University of the Negev
P.O.B. 653, Beer-Sheva 84105, Israel.
[2]Nitrogen Fixation Laboratory, Univ. Sussex, Brighton BN1 9RQ, UK.

The conveners in their introductions used the Rees model of the nitrogenase protein complex and its metal clusters to formulate the questions that should be addressed in the discussion. These included the site of N_2 binding and the nature of metal-bound intermediates, the Russian view favouring a diazene or hydrazine derivative stabilised by electron donation from a metal cluster. It was stressed that although it is generally accepted that substrate reduction occurs on the FeMo-cofactor, there is currently no data to show whether Fe or Mo or both metals are involved in N_2 reduction. A key question was the role of homocitrate and the influence of protein environment, in particular hydrogen bonding and electrostatic interactions with the FeMo-cofactor. The mechanism of ATP hydrolysis and energy transduction via the Fe-protein/MoFe-protein interface should be discussed with reference to other systems such as actino-myosin, kinesin and G-proteins. Data to be presented by Dr. Lowe in his lecture (see also his article in this book) shows that phosphate is released from the nitrogenase complex at a rate that is very similar to that measured for these analogous energy transducing, two protein, systems (k for phosphate release in the range 15 to 22 s^{-1}). It is currently not clear how the energy of ATP hydrolysis is used although an attractive hypothesis is that protein conformation changes originating in the Fe-protein transmitted to the MoFe-protein via the interface, modulate the protonation state and redox potential of the FeMo-cofactor.

The aim of the round-table discussion was to integrate the chemical and enzymological approaches to these questions in order to achieve a consensus on the mechanism of nitrogenase catalysis, with a view to future experimental verification.

Dr. Denisov (Russia) reported the reduction of N_2 to N_2H_4 and NH_3 by individual and mixed hydroxides of Mo(III), V(II), Cr(III) and Nb(III) in aqueous methanol. Although these systems have different catalytic activities, common features include higher activity at higher pH, an optimal H_2O concentration and a significant isotope effect (1.6) when H_2O is replaced by D_2O. Deprotonation of a metal hydroxide followed by the formation of a metallo-hydride and subsequent reduction of bound N_2 was proposed as key steps in the mechanism.

Prof.Coucouvanis (USA) described studies with Fe/Mo/S and Fe/V/S synthetic clusters with the heteroatom (Mo or V) in a coordination environment similar to that

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found in the nitrogenase cofactors. The catalytic reduction of hydrazines ($RNHNH_2$, R=H, Ph, CH_3) to NH_3 and RNH_2 was effected only with simple cubanes and involved exclusively heterometals (Mo or V). Protonation was necessary prior to reduction and the rate was enhanced by a citrate ligand on the heterometal. Loss of a protonated carboxylate ligand of the citrate to generate a site for substrate binding was proposed. After reduction of the cluster the protonated carboxylate could act as a general acid catalyst for substate reduction and protonation. **Dr.Lowe** (UK) drew parallels between this system and that recently published by **Dr.Pickett** (UK) on $[MoH_2(CH_3COO)(dppe)_2]^+$ which on reduction and loss of acetate binds N_2. Both systems indicate possible roles for homocitrate at the active site of nitrogenase. **Prof. Shilov** (Russia) commented that he thought that it was unlikely that Mo was the active center for N_2 reduction, particularly if N_2 is proposed to displace a bound carboxylate on Mo(IV).

Prof. Coucouvanis then described how acetylene reduction on a Mo-single cubane cluster takes place at both the Fe and Mo. This indicates that there could be multiple sites for acetylene reduction on the FeMo-cofactor as is also suggested by the site-directed mutagenesis studies of Newton (see below) and CO inhibition results of Hales (see elsewhere in this book). **Prof. Smith** (UK) reported on the binding of cyanide to FeMo-cofactor isolated from *K.pneumoniae*. The cyanide probably binds to sites on the Mo and Fe which are vacated by histidine and cystein groups in the protein on extraction. These sites are, therefore probably not the site at which HCN binds in the active enzyme. Fe and Se K-edge EXAFS data had demonstrated that selenophenol and, by analogy thiophenol bind to an Fe atom in the FeMo-cofactor. This is also probably the terminal Fe coordinated by cystein in the protein. Reaction of FeMo-cofactor with benzene or toluene dithiol results in degradation of the cofactor by loss of sulphur and activity to yield a red compound containing Mo and Fe in a ratio 1:6. This compound can be oxidised by controlled exposure to air to yield a blue compound. Both red and blue compounds are paramagnetic.

Dr. Shestakov A.F. (Russia) presented theoretical calculations that showed that in the resting state, the FeMo-cofactor contains metal ions with oxidation states Mo(IV)4Fe(II)3Fe(III). The overall charge of the cofactor is therefore zero and +1 if one assumes protonation of a bridging sulphide due to the strong H-bond with Arg-96 as indicated in the Rees structure of Av1 and the mutagenesis studies described below by Newton. This charge distribution removes the approximate C_{3V} symmetry of the FeMo-cofactor and explains the five different types of Fe atoms observed by ENDOR-spectroscopy (Hoffman, USA). Assuming strong antiferromagnetic coupling between the Fe atoms on the edges of the Fe_6 prism then it is possible to assign the cofactor spin states as: resting S=3/2, oxidised S=0, reduced S=1 provided the capping Fe atom is Fe(III). With regard to the site of N_2 binding **Dr.Shestakov A.F.** suggested N_2 interacting with a Fe_4 face along its diagonal or N_2 half-penetrating through the Fe_4 face. The first model is also clearly possible for acetylene binding but not for ethylene. The second model is analogous to the known cage complex containing a $[N_2Sm_2Li_4]^-$ cluster.

Prof. Newton (USA) commented that the polypeptide environment of the FeMo - cofactor plays an important role in modulating its catalytic and spectroscopic properties. Individual substitutions by Lys at αArg-96, αArg-359 or αGln-191, all of which have putative hydrogen bonds to the Mo-subcluster of the FeMo-cofactor, introduce carbon monoxide inhibition of H_2 evolution activity by these MoFe-protein variants. Disruption of the hydrogen bond to a central bridging sulphide in the cofactor by mutating αHis-195 to Asn results in complete loss of N_2 reduction while retaining acetylene reduction and hydrogen evolution activities. The αHis-195 Gln variant, for which modelling indicates the glutamine can form a hydrogen bond to the bridging sulphide, does bind N_2 as judged by inhibition of hydrogen evolution but does not reduce N_2 to ammonia. **Dr. Henderson** (UK) reminded the meeting that protonation of clusters can also occur at sites other than terminal thiols or bridging sulphides. His work had shown that protonation at the face of the cluster was a distinct possibility.

Prof. McKenna (USA) emphasized the need to develop new enzymological information in order to elucidate the chemical pathways of nitrogenase substrate reductions. A familiar thermodynamical calculation demonstrates that among hypothetical $2e^-/2H^+$ reduction structures on the pathway from N_2 to NH_3, N_2H_2 in the form of diazene (HN=NH) is much less stable than N_2H_4. There is no conclusive evidence for a diazene intermediate in N_2 reduction by nitrogenase, and arguments have been propounded that the enzyme in fact avoids it by transferring electrons and protons in a ratio other than two, at least at this step; or alternatively that the N_2H_2 intermediate does occur, but as its tautomer, aminonitrene (also presumed to be in a site-bound form). However, it is also worth noting that a thermodynamical calculation for the energy of "free" diazene vs. (N_2 + H_2) neglects active site binding that could lower the energy of diazene: the enzyme could seek to stabilise diazene as part of its catalytic strategy. Free diazene is labile, and has not been demonstrated to be a substrate of nitrogenase. New work of McKenna's laboratory has established that *trans*- and *cis*-dimethyldiazene (CH_3-N=N-CH_3) are substrates of nitrogenase. The results prove that nitrogenase is capable of cleaving an acyclic, symmetrical N=N bond, like that in diazene. The *cis* isomer is more effectively reduced than the *trans* isomer, but has a 100-fold larger K_m than N_2 or diazirine (a compact 3-membered ring containing a strained N=N bond) indicating that steric and perhaps also electronic effects significantly modify binding and reduction of these substrates.

Dr Druzhinin (Russia) described a new photochemical electron donor system for nitrogenase that utilises eosin as a photosensitiser and NADH as the electron donor. The anion radical form of eosin reacts with oxidised Av2 to support nitrogenase catalysed reduction of acetylene. A two fold increase in rate relative to that obtained using dithionite as reductant was reported. The Sussex group commented that this did not necessarily mean that with eosin as reductant, protein complex dissociation was no longer rate limiting since flavodoxin induces a similar enhancement under the assay conditions used. This is because under these conditions of low electron flux, reduction of oxidised Fe-protein, not protein complex dissociation, is rate limiting. The eosin data may only mean that it is a faster reductant than dithionite ion and probably reduces oxidised Fe-protein with 2MgADP bound at a rate comparable to that of flavodoxin.

Dr. Huang (China) showed how nitrogenase and FeMo-cofactor are currently prepared in China and outlined future experiments on isolated cofactor. He described procedures for the replacement of Mo by a number of transition metal ions including Mn(II) which gave characteristic EPR spectra.

In summing up, **Dr. Thorneley** said there is clearly a pressing need to develop time-resolved spectroscopic methods that would allow the direct detection and characterization of enzyme-bound intermediates in N_2 reduction. He was about to commence such studies himself in collaboration with Dr.C.Wharton (UK) who had recently built a high-resolution FTIR stopped-flow spectrometer. Dr. Thorneley hoped to have the results available for the next congress in Paris.

ROLE OF POLYSACCHARIDES AND OTHER GLYCOCONJUGATES IN NITROGEN-FIXING BACTERIAL-PLANT ASSOCIATIONS.

DAZZO F.B.[1] and ROLFE B.G.[2] (*Conveners*).
[1]Department of Microbiology, Michigan State University, East Lansing, Michigan 48824 U.S.A.
[2]Plant-Microbe Interactions Group, Research School of Biological Sciences, Australian National University, Canberra, Australia

All plant-associated nitrogen-fixing bacteria produce polysaccharides and other glycoconjugates in order to successfully maintain their ecological niche. The most thoroughly examined glycoconjugates are those made by the root-nodule bacteria, which synthesize a diversity of glycoconjugates necessary for successful development of their nitrogen-fixing symbioses with legumes. Recent work in this field has revealed several new classes and elucidated structures of complex glycoconjugates made by plant-associated diazotrophs, new information on the genetic analysis of their biosynthetic pathways, new technologies to examine the complex structures and *in situ* interactions of polysaccharides with host plants, and further insights into their biological activities and functions during symbiotic development. Participants at this roundtable session will highlight these advancements of knowledge (briefly summarized below) and address future research directions to further elucidate the roles of glycoconjugates in development of nitrogen-fixing associations between bacteria and plants.

Structural diversity and novel classes -- *Rhizobium* produces many different types of glycoconjugates, including multiple types of secreted acidic heteropolysaccharides (CPS and EPS) and native oligomers with the same repeat unit structures, multiple glycoforms of lipopolysaccharides, multiple classes of β-homoglucans (β-1,2-, β-1,3-, β-1,4-, β-1,6-), peptidoglycan, and two families of small glycolipids: chitolipooligosaccharides and glycosyl diacylglycerols. Of these, the lipopolysaccharides remain the most complex challenging structures to solve. Topics for discussion will include recently completed structures of *Rhizobium* glycosyl diacylglycerols, lipid A, two lipopolysaccharide core oligosaccharides, and the first O-antigenic oligosaccharide repeat unit of lipopolysaccharide.

Genetic analysis of glycoconjugate biosynthesis -- The genes involved in biosynthesis of the major acidic heteropolysaccharides have been most thoroughly examined in *R. meliloti*, and of lipopolysaccharides in *R. leguminosarum* bv. *viciae*. Additional topics for discussion will include the identification of novel genes for synthesis of β-glucans in *B. japonicum*, of EPS in *B. japonicum*, *R. astragalus*, *R. leguminosarum* bv. *viciae*, and *R. sp.* (*Acacia*), of LPS in *R. fredii*, *R. leguminosarum*

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bvs. trifolii and *viciae*, *R. meliloti*, and *Azospirillum brasilense*, and of the link between purine auxotrophy and glycoconjugate synthesis / function in *Rhizobium*.

Environmental-induced structural modifications of glycoconjugates -- Two well-known examples on this topic include the exuded clover root enzymes (Rlt-CPS polarase) that degrade the capsular polysaccharides of *R. leguminosarum* bv. *trifolii*, and hypoosmotic induction of periplasmic β-1,2-glucan synthesis in Rhizobiaceae. Additional topics for discussion include induction of novel sulfoglycosyl diacylglycerols and EPS in *Rhizobiaceae* bacteroids, *nod*-dependent sulfation of LPS in *R. meliloti*, and other alterations in LPS of *R. etli* and *R. meliloti* induced by root exudate and NaCl.

Functional roles of bacterial glycoconjugates in symbiosis with plants -- Analysis of mutant phenotypes and/or bioassays of biological activity have shown that representatives of each known class of *Rhizobium* glyco-conjugates are involved in one or more developmental stages of the nitrogen-fixing symbiosis with legumes. Also, new activities of glycoconjugates involved in other plant-associated bacteria are now coming to light. Topics relevant to this section of the roundtable include the evidence that glycoconjugates serve active roles as biosignals / elicitors of (lectin-mediated) positive host responses, active roles as negative regulators of inhibitory host defense responses, and passive roles providing physical protection or molecular mimicry to mask the invasive bacterium and evade its recognition by the host. Additional topics for discussion include recent evidence that alterations in EPS and LPS affect host range of nodulation for *R. fredii* and *R. sp.* (*Acacia*), wheat proteins involved in bacterial attachment are induced by LPS from *Azospirillum*, wheat lectin induces several metabolic changes upon binding to *Azospirillum*, EPS from *Azospirillum* induces morphogenetic responses in wheat root hairs, enzyme cytochemistry reveals the *in situ* cellulosic nature of microfibrils associated with *Agrobacterium* and *Rhizobium* attached to their host roots, laser scanning confocal microscopy reveals overexpression of LPS epitopes by *R. leguminosarum* bv. *trifolii* enclosed within shepherd crooks of white clover root hairs and at/in rhizosermal cells of maize, and the acoustical properties of *Rhizobium* exopolysaccharides reveal new insights into their ability to act as molecular biosensors.

Further information on the structures, genetic regulation and role(s) of polysaccharides and other glycoconjugates in nitrogen-fixing bacteria-plant associations has been published (Napoli et al., 1975; Abe et al., 1982; Dazzo et al., 1982; Miller et al., 1986; Djordjevic et al. 1987; Hollingsworth et al., 1988; Carlson et al., 1988; Hollingsworth et al., 1989; Michiels et al., 1989; Gray, Rolfe, 1990; Dazzo et al., 1991; Patseva, Kosenko, 1991; Kijne, 1992; Orgambide et al., 1992a; Orgambide et al., 1992b; Kannenberg et al., 1992; Streeter et al., 1992; Lopez-Lara et al., 1993; Noel, 1993; Puhler et al., 1993; Whitfield, Valvano, 1993; Cedergren, Hollingsworth, 1994; Wang, Hollingsworth, 1994; Leigh, Walker, 1994; Bhat et al., 1994; Carlson et al., 1995, Mateos et al., 1995; plus many more) and are presented in many posters at this international conference (see proceedings abstracts by [1st author] Antonyuk, Becker, Bhagwat, Buendia-Claveria, Cedergren, Chumakov, Dunelli, Garcia de los Santos, Ivashina, Karpunina, Kosch, Kosenko, Lagares, Lloret, Petrova, Philip-Hollingsworth, Priefer, Song, and Yu).

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NITROGEN FIXATION IN PHOTOTROPHIC BACTERIA

GALLON J.R.[1] & WOLK C.P.[2] (*Conveners*).
[1] School of Biological Sciences, University of Wales Swansea, Singleton Park, Swansea, SA2 8PP, U.K. [2] MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824, U.S.A.

N_2-fixing phototrophs (photosynthetic bacteria and cyanobacteria) occur in terrestrial, freshwater and marine environments from polar regions to the tropics. Some of these organisms contribute substantially to global N_2 fixation. For example, blooms of the marine cyanobacterium *Trichodesmium* may cover 90000 km^2 of the tropical ocean (Dupouy *et al*, 1988) and fix as much as 5.4×10^{12} g of N per year (Carpenter, Capone, 1992), perhaps 4% of the total N_2 fixed on Earth (see Paul, 1978, and papers cited therein). In addition, *Nodularia* and *Aphanizomenon* spp form extensive annual blooms in the Baltic Sea (see, for example, Kahru et al., 1994). Cyanobacterial symbionts of marine diatoms such as *Hemiaulis* spp and *Rhizosolenia clevei* may also contribute a great deal locally to oceanic N_2 fixation. (Villareal, 1991; 1992; 1994). Finally, the discovery of marine copepods bearing intestinal *Chromatium* spp and able to reduce acetylene (Proctor, 1995; personal communication to JRG) implies that marine photosynthetic bacteria may also make a considerable contribution to global N_2 fixation, perhaps up to 1% of the total. Photosynthetic diazotrophs therefore make major contributions to N_2 fixation on Earth and, as such, deserve intensive study.

In this Round Table Discussion, consideration of N_2 fixation by phototrophs centred on: (a) regulation of N_2 fixation (with particular emphasis on similarities and differences between photosynthetic bacteria and cyanobacteria), (b) heterocyst differentiation and (c) symbioses. In addition to the Conveners, participants included: E.K. James (St Andrews), B. Masepohi (Bonn), S. Nordlund (Stockholm), S. Shestakov (Moscow), Y.Q. Wu (Shanghai) and V. Zinchenko (Moscow).

Nordlund: Nitrogenase activity in *Rhodospirillum rubrum* is regulated at the metabolic level by reversible ADP-ribosylation of dinitrogenase reductase in response to external effectors such as NH_4^+ and energy supply (light). The enzymes catalyzing this modification and demodification (DRAT and DRAG, respectively) have been characterized in detail by Ludden and coworkers. However the internal signal linking external effectors to the activities of DRAT and DRAG has not yet been identified. A model for this signal transduction is proposed in which the concentration of oxidized NAD regulates activity of DRAT whilst energization of the chromatophore membrane influences DRAG activity.

Gallon: A number of N_2-fixing cyanobacteria possess two electrophoretically distinguishable forms of dinitrogenase reductase (see Gallon, 1992). Although this is

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reminiscent of the situation in purple photosynthetic bacteria, ADP-ribosylation of dinitrogenase reductase has not been demonstrated, either in *Anabaena variabilis* (Durner *et al*, 1994) or in *Gloeothece*, even though the latter contains proteins that cross-react immunologically with anti-DRAT and anti-DRAG (Du et al., 1994). Modification of cyanobacterial dinitrogenase reductase is stimulated by oxidative stress or addition of NH_4^+ and, in some cyanobacteria, parallels the pattern of nitrogenase activity seen under alternating light and darkness. In other cyanobacteria, however, this pattern of activity reflects synthesis and degradation of both nitrogenase proteins rather than modification and demodification of an unchanging intracellular concentration of dinitrogenase reductase (Du, Gallon, 1993; also see Gallon et al., this volume). In *A variabilis*, dinitrogenase reductase can be ubiquitinylated *in vitro*, (Durner, Böger, 1995), suggesting that, as in eukaryotes, targeting of cyanobacterial proteins for proteolytic degradation my involve ubiquitin-tagging.

Zinchenko: Insertion inactivation of the *ntrC* gene in *Rhodobacter sphaeroides* causes total repression of both the *glnBA* operon and structural *nif*-genes. Moreover, these mutants grow slowly under all tested conditions and are deficient in their use of carbon sources that are metabolized via pyruvate. Metabolism of nitrogen and carbon, along with energy generation, may therefore be coordinately regulated in R *sphaeroides*.

Wu: All of fourteen GOGAT$^-$ mutants, isolated from *Rhodobacter sphaeroides* 601 by mutagenesis with Tn5 or with NTG, were Nif$^-$ and Ntr$^-$ when grown with malate as a carbon source. A cosmid bearing *R sphaeroides* DNA, and homologous with both *E coli gltBD* and *glt* from *A sesbaniae*, restored GOGAT and nitrogenase activities, along with the ability to use several nitrogen substrates. The *glt* gene has been mapped in *R sphaeroides* 601. One of the GOGAT$^-$ mutants (204) had a Nif$^+$ phenotype when grown in medium containing pyruvate (see Wu *et al*, this volume). Intracellular glutamine in this mutant was 16 times more concentrated than in the wild type and nitrogenase activity appeared as intracellular glutamine became depleted. Glutaminase and alanine dehydrogenase activities were induced in cells by pyruvate, so the appearance of nitrogenase under these conditions may result from direct decomposition of intracellular glutamine along with amination and transamination of pyruvate.

Masepohl: The *fdxH* gene from *Anabaena* sp PCC 7120 encodes a [2Fe-2S] ferredoxin. An *fdxH*$^-$ mutant exhibited only 20-25% of the nitrogenase activity of the wild-type. En route to analysing the role of chaperones in *nif* gene expression, the *dnaK* (*hsp70*) gene has been cloned from *Anabaena variabilis* ATCC 29413. Nucleotide sequence analysis of a 1.3-kb DNA region carrying transposon Tn5-1087b from *Anabaena* 7120 mutant YC16 (obtained from C.P. Wolk) revealed the presence of 17 copies of a novel 37-bp repeated DNA sequence designated LTRR (long tandemly repeated repetitive).

Shestakov: (*Coworkers: F. Kamilova, Tashkent; L. Mikheeva, G. Grigorieva, N. Belavina, L. Shakhnabatian, Moscow; A. Ernst, P. Böger, Konstanz; L.J. Stal, Amsterdam; J.R. Gallon, Swansea*) The regulation of N_2 fixation and the mechanisms of protection of nitrogenase against O_2 in unicellular cyanobacteria is being studied by mutagenesis of *Gloeothece* spp PCC 6909 and CCAP 1430/3 (which are capable of aerobic N_2 fixation) and *Synechocystis* sp BO 9201 (which fixes N_2 only microaerobically). Mutants resistant to oxidative stress and to inhibitors of glutamine synthetase or (in the case of *Gloeothece*) incapable of aerobic N_2 fixation have been isolated. In addition, a cosmid library of *Synechocystis* DNA has been constructed and a system for gene transfer by conjugation, based on the pDU1 replicon, has been developed.

Wolk: Highly similar nucleotide sequences are present 3' from, and near, *Anabaena* genes (*hetC*, *devA*, *hepA*) required for the Fox$^+$ phenotype (ability to *fix* nitrogen in the presence of *oxygen*; I. Khudyakov and C.P. Wolk, unpublished). We are trying to determine whether this "Fox Box" plays a role in developmental regulation. To function, it might require a protein, such as *E. coli* HU, to help maintain a loop in DNA at the 5' end of these genes. Consistent with this idea, we have shown that a mutation in the *Anabaena* equivalent of HU prevents heterocyst formation (idem).

James: (*Coworkers: P. Rowell, Dundee; P. Nixon, London; R.M.M. Crawford, St Andrews; W.B Silvester, Waikato*) The 33-kD extrinsic protein of the water-splitting complex and the D1 and D2 proteins required for subsequent transfer of electrons to plastoquinone have been located by immunogold labelling in *Anabaena cylindrica*, a free-living *Nostoc* sp and symbiotic *Nostoc* from *Gunnera manicata*. D2 was particularly abundant in vegetative cells but this protein, and the 33-kD O_2-evolving protein, were found in both heterocysts and vegetative cells. D1 was the least apparent protein and found only in vegetative cells. Heterocysts of free-living and symbiotic cyanobacteria may therefore retain much of the PSII apparatus, though whether this has a role in these cells is unknown.

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OXYGEN AS THE MAJOR REGULATING FACTOR IN SYMBIOTIC NITROGEN FIXATION.

ATKINS C.A.(1) AND MINCHIN F.R.(2) *(Conveners)*.
(1) Department of Botany, University of Western Australia, Nedlands, 6009, Australia
(2)IGER, Plas Gogerddan, Aberystwyth, Dyfed SY23 3EB, UK.

There is now considerable evidence for the existence of a variable barrier to O_2 diffusion in legume nodules. However, there is still controversy as to its location, structure and mode of operation. Furthermore, we are only starting to address questions concerning the sensing of pO_2 changes and the transduction of such signals. Then again, in order to exploit O_2 regulation we have to understand how it is used in the legume symbiosis.

Question 1 - Where is the O_2 barrier and how does it work?

Steve Hunt (Queen's University, Canada) pointed out the need to distinguish between a short-term (5min), "physiological barrier" and a long-term (hours/weeks) "developmental barrier". For physiological barriers in the cortex there is little evidence for variations in osmotica, whilst that for changes in size or occlusion of intercellular spaces has been challenged by cryo-planning studies. Recent mathematical models predict that infected cells have intrinsic mechanisms for regulating their O_2 status. These models suggest that a greater pO_2 is maintained in the central region than previously envisaged and challenge the dogma that nodule O_2 regulation is exclusively in the cortex.

Jean Jacques Drevon (INRA - Montpellier, France) presented evidence that, in soybean nodules, permeability decreases induced by supra-optimal pO_2 are associated with a contraction of inner cortex cells. Differences in velocity of permeability decreases, compared to increases, and sensitivity to shock suggest a reversible mechanism similar to that of legume pulvini cells; ie. water movement driven by K^+ fluxes. This is supported by supra-optimal pO_2-induced decreases in the electrical potential of inner cortex cells and, indirectly, by ABA-induced decreases in nitrogenase activity. The presence of a cyanide-insensitive respiratory pathway in inner cortex cells suggests that their mitochondria may be sensors for changes in malate or O_2 concentrations.

Euan James (St.Andrews University, UK) showed variations in glycoprotein occlusions of intercellular spaces in the mid-cortex region. This could provide either a

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physiological or a developmental barrier in soybean nodules. Moreover, immunogold labelling and ELISA studies with lupin nodules have shown that glycoprotein excretion can increase within 15 min after exposure to 50% O_2. In addition, immunogold labelling has demonstrated a pathway of active intracellular production, followed by extrusion of glycoprotein across cell walls and into intercellular spaces within this time period. There are a number of other materials in cortical intercellular spaces but their roles (if any) in O_2 regulation have yet to be established.

Question 2 - How is pO_2 sensed and the signals transduced?

Alexey Topunov (A.N. Bach Institute, Moscow) reported on studies of adenylate energy charge (AEC) within infected cells of soybean nodules. This decreases with low pO_2, stem girdling and nitrate, and increases with high O_2 and reduced temperature. Separation of rapidly frozen nodules into plant and bacteroid fractions has been achieved by non-aqueous fractionation. The plant fraction has a lower AEC than the bacteroid fraction, is more sensitive to changes in pO_2 and seems to be the site of O_2 limitation; whilst the bacteroids are C-limited. It was suggested that the plant fraction AEC is the O_2 sensor for regulating nodule permeability; infected cell O_2 concentration is adjusted to maintain adenylated pools at levels which limit nodule metabolism.

Kathy Schuller (Flinders University, Australia) reported that PEPC assays of nodules from decapitated plants show increased sensitivity to malate inhibition compared to control plants. A decreased flux of sucrose to the nodules could produce a decrease in G-6-P concentration (an allosteric activator of PEPC) resulting in decreased activation of PEPC and/or PEPC-protein kinase. This would lead to decreased malate production, C-starvation of bacteroids and reduced N_2 fixation. The resultant lower fluxes of ureides from the central region could act as a "signal" for a decrease in nodule O_2 permeability. An increase in external pO_2 would reverse this through increasing ATP/ADP ratios and hence PEPC-protein kinase activity.

Richard Parsons (University of Dundee, UK) suggested that nodules "sense" the nitrogen status of the plant and alter their metabolism to meet the plant demands. Nodules contain high levels of soluble amino acids and ureides, but not all these compounds are manufactured in the nodule; some are delivered in the phloem sap. In soybean, the levels and activity of amino compounds have been followed over time after the addition of $^{15}NO_3^-$ to the roots. Asparagine appears first in the leaves and subsequently in the roots, xylem and nodules. It accumulates in the nodule and may act directly to regulate N_2 fixation by influencing the O_2 diffusion barrier; via modulation of the activity of key enzymes or by acting as am osmoticum.

Question 3 - How can we exploit O_2 regulation?

Gina Hernandez (UNAM-Cuernavaca, Mexico) reported on alterations in O_2 regulation of *Rhizobium etli* nitrogen fixation genes through the expression of a heterologous bacterial globin gene: the *vgb* gene from *Vitreoscillar*. A *R. etli* strain bearing the pHRO$_4$(+*vgb*) plasmid shows a higher *ex planta* expression of the *nifHa* and *nifHe* reiterated genes in very low O_2 concentrations (0.25-0.75%). This strain also shows increased nodulation and nitrogen fixation at early stages of the symbiosis with *Phaseolus vulgaris* which, under controlled conditions, leads to improved plant growth and earlier pod production. This approach provides a system to modulate the intracellular O_2 concentration and regulate the expression of essential symbiotic genes.

Kerry Walsh (University of Central Queensland, Australia) addressed the role of the O_2 barrier in nitrogenase protection. A number of environmental stresses (eg. nitrate, detopping, $Ar:O_2$) have been shown to affect nodule activity primarily through an O_2 limitation; as measured by oximetry and demonstrated through recovery of activity with increased pO_2. However, whether or not these changes are secondary responses to C limitation has yet to be established. C partitioning to the nodule appears to be dramatically halted by 100% O_2, suggesting that phloem unloading is responsive to metabolic signals. In other stresses (eg. temperature, water stress) changes in nodule permeability seem to occur after changes in metabolic rates within the infected region.

Ulei Hartwig (Federal Institute of Technology, Switzerland) pointed out that nodule activity could be regulated by the physiology of the whole plant. Since nutrient uptake is governed by plant growth, inhibition by any environmental constraint will reduce nutrient assimilation to below that of non-stressed plants. This might be particularly important with the organic products of N_2 fixation, because ammonia is highly toxic and plants must balance fixed N input against their N assimilation requirements. One obvious way to do this is through the variable O_2 diffusion barrier of the nodules, by using a physiological trigger. Free ammonia is unlikely to be the trigger, but enhanced levels of amino acids or organic acids can affect the regulation of N_2 fixation.

At the end of this discussion session there seemed to be general agreeement that the diffusion "barrier" was a complex of biochemical and physical features encompassing both the infected region and the cortex. The next important step is to understand the interrelationships between these processes. The question of O_2 sensing and signal transduction still remains largely unanswered, although several interesting ideas are now emerging which should be amenable to experimental testing at the biochemical/genetical level. The discussion also provided hope for the exploitation of O_2 regulation at the level of symbiotic gene expression and whole plant control of nodule activity.

NITROGEN FIXATION AND SUSTAINABLE AGRICULTURE

LADHA[1] J.K AND RUPELLA[2] O.P. (*Conveners*),
[1]International Rice Research Institute, Philippines.
[2]Agronomy Division, ICRISAT Asia Center, Patancheru 502 324,
Adhra Pradesh, India.

From early 1980's decline in funds for biological nitrogen fixation research was observed particularly for field applications. This was echoed in some international meetings in the 1980's (e.g. 6-th International Symposium on N_2 Fixation, Corvallis, August 4-10, 1985). Some scientists now believe that this research area will soon want getting its due attention and funding. One important input, however, needed is to demonstrate the on-farm use of BNF in sustaining and enhancing agricultural production. This is important for both legumes and cereals.

This roundtable session was devoted to discussions on the role of BNF in agricultural sustainability. Several brief reports were presented on diverse aspects of BNF covering legumes to cereals which were augmented with discussions within the framework of agricultural sustainability.

Biological nitrogen fixation *vs* chemical fertilizer

Over the past 25 years the farmers have become increasingly dependent on chemical sources of Nitrogen. There are signs of environmental degradation especially in continuous cereal cropping which is being attributed to heavy use of chemical nitrogen. It was argued that chemical fertilizer and BNF are not incompatible and rather they are complementary. There is need to develop ways to integrate BNF and fertilizer N to obtain sustainable agricultural systems.

Improving BHF in non-legumes

Substantial yield improvements in cereals such as rice, wheat and maize have taken place during last decades. This has also resulted in tremendous use of chemical N fertilizer which is based on non-renewable energy resource. There is an outstanding need to improve and/or extend symbiotic N_2 Fixation in cereals.

Novel associations both naturally occurring and artificial symbiosis between diverse N_2 fixers including rhizobia and non-leguminous crops such as rice, maize and wheat were presented. This area of research seems to have a lot of exciting and promising opportunities. However, there was a note of caution with regard to the claims made.

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It was suggested that adequate and proper techniques should be employed to confirm the observations made.

It was felt that although substantial progress in the area of nonlegume-microbe interactions has been made, much more research is still needed to better understand the system before the system can be fully exploited in agriculture.

Improving BNF in legumes and sustainability of cropping systems

Interactions in multidisciplinary teams suggest that some simple questions in relationships between nodulation (or BNF) and yield in legumes are not simple to explain because of obvious interaction between soil mineral N level, host plant and BNF. This area needs more attention.

In the legume-*Rhizobium* symbiosis a host plant seems to play a stronger role than the symbiont. Because under same environmental conditions two cultivars of a legume nodulate greatly differently even when exposed to same rhizobial population. Plants with greatly different nodulation capacities have been obtained even in released cultivars. This has important implications on rhizobial strain selections based on their interactions with host-plant.

There are excellent opportunities for integrating dual or multipurpose legumes (grain, forage and green manure) in cereal cultivation. Papers presented clearly show that the legumes in cropping system contribute significantly to the maintainance of soil fertility and productivity.

Participants:
Baldensperger J. (The Netherlands), Bashan Y. (Mexico), Bockman (Norwey), Hallberg B. (Mexico), Hardarson G. (Austria), Hoflich G. (FRG), Hopper W. (India) Imsande J. (USA), Kozhemyakov A.P. (Russia), Kruglov Yu.V. (Russia), Malik K.A. (Pakistan), Mandimba G.R. (Congo), Meletzus D. (USA), Mpepereki S. (Zimbabwe), Sadowsky M.J (USA), Selenska-Pobell S. (FRG), Tripathi A.K. (India), Yanni Y.G. (Egypt) and others.

REPORT FROM THE ROUNDTABLE ON RHIZOBIUM TAXONOMY

LINDSTRÖM [1] K., VAN BERKUM[2] P., GILLIS[3] M., MARTÍNEZ[4] E., NOVIKOVA[5] N. AND JARVIS[6] B.
[1]Department of Applied Chemistry and Microbiology, P.O.Box 27, FIN-00014 University of Helsinki, Finland, [2]USDA-ARS, HH-19 Bldg 011 BARC-West, Beltsville MD 20705, USA, [3]Microbiological laboratory, University of Gent, KL Ledeganckstraat 35, B-9000 Gent, Belgium,[4]Center for Nitrogen Fixation, UNAM, Apdo postal 565-A, Cuernavaca, Mor, Mexico 0052, [5]All-Russia Institute for Agricultural Microbiology, Podbelsky Shosse 3, St. Petersburg-Pushkin 8, 189620 Russia and [6]Department of Microbiology and Genetics, Massey University, Private Bag, Palmerston North, New Zealand

INTRODUCTION

During recent years, there has been an explosion in the field of *Rhizobium* taxonomy (*Rhizobium* is defined here as all root and stem nodulating bacteria). In particular, recent emphasis has been on tropical rhizobia and the vast biodiversity of this group of bacteria has become evident. Molecular methods nowadays offer sophisticated means of constructing phylogenies for bacteria and they also facilitate rapid and accurate genomic fingerprinting at the strain level. The Subcommittee on the Taxonomy of *Agrobacterium* and *Rhizobium* is a forum for discussions and recommendations in the field of taxonomy. However, the committee cannot make decisions as to whether new species and genera should be designated and named, since articles proposing those undergo normal peer review and follow standard publication policies. During the conference, a roundtable workshop covering recent advances in *Rhizobium* and *Agrobacterium* taxonomy was organized.

NEW SPECIES

The following species and genera have been proposed since the last meeting of the subcommittee in Cancun in 1992.

Rhizobium etli (Segovia et al., 1993) is a bean-nodulating species on the same major phylogenetic branch as *R. leguminosarum*. This species is distinguished from other species at a molecular level by DNA hybridization of total genomic DNA, multilocus enzyme electrophoresis and 16S ribosomal gene sequences. No phenotypic tests that are diagnostic have been described. It was agreed that this taxon fulfills present criteria for new species, although requests for a minimal phenotypic description were formulated.

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Sinorhizobium saheli and ***S. teranga*** (de Lajudie et al., 1994) nodulate *Acacia* and *Sesbania* trees in Africa. The taxa were appropriately described by auxanography (metabolic testing), protein patterns in SDS-PAGE, DNA hybridization and sequencing of full length 16S ribosomal genes of the type strains. The proposed genus name will be dealt with below.

R. ciceri (Nour et al., 1994) is a group of rhizobia nodulating chickpea. The objective of the study describing the chickpea rhizobia was to provide better genetic characterization of rhizobia nodulating *Cicer arietinum* and to include a description of its diversity. During the debate it became clear that the chickpea rhizobia are more diverse than the data in the paper would indicate. The phylogenetic position of the chickpea rhizobia, based on 16S sequence analysis, is close to that of *R. loti* and *R. huakuii*. Full descriptions of the diversity within and among groups of rhizobia nodulating chickpea are essential because potentially many new taxa share the phylogenetic position with *R. loti*. Therefore, it was concluded that the genetic analyses presented were incomplete and that the populations had not been sampled enough to merit one group of chickpea rhizobia to be described as a separate species *R. ciceri*.

The name ***R. tianshanense*** was proposed by Chen et al. (1995) for a group of rhizobia with unusual growth properties originating in an arid environment in Northwestern China. Although the group was identified by numerical taxonomy, diagnostic phenotypic traits and symbiotic properties were not satisfactorily described. Its DNA homology with *R. huakuii* was above 60% and the partial sequence of the 16S gene (260 bp of a variable region sequenced after amplification with primersY1 and Y2) was identical to that of *R. ciceri*. For these reasons there is doubt as to the taxonomic status of the proposed species, which should, like *R. ciceri*, be reexamined and compared with other new and emerging species on the *R. loti* phylogenetic branch.

R. hainanensis was proposed by Chen et al. at the *International Symposium on Rhizobium Diversity and Taxonomy* held in Wuhan, China in October 1994. The group was identified by numerical taxonomy and it contains 13 strains isolated from 13 different plant species growing on the tropical island Hainan in China. DNA homology within the group, as determined by a spectrophotometric assay, was above 70% and the homology with other taxa below 30%. However, the partial sequence of a 16S ribosomal gene was identical with that of *R. tropici*. *R. tropici* consists of two subspecies, A and B, which have 36% DNA homology between them, but which are not distinguished at the species level. Because *R. hainanensis* was only compared with the type strain of *R. tropici*, DNA homology studies with a larger number of *R. tropici* strains are required in order to clarify the relationship between *R. hainanensis* and *R. tropici*. The validation of the name *R. hainanensis* for this new group is strongly discouraged.

Bradyrhizobium liaoningensis was proposed at the Wuhan meeting by Xu et al. to describe extra slow-growing soybean strains nodulating Chinese rhizobia. The criteria used to separate this taxon from other bradyrhizobia were results from numerical taxonomy, DNA hybridization and partial 16S sequencing studies. The DNA relatedness with other bradyrhizobia was 19-30% and there was a difference of seven bases between the 16S sequences of *B. liaoningensis* and *B. japonicum*, but no comparison with *B. elkanii* was made. In view of the sequence variability within *Bradyrhizobium* 16S rRNA genes, these results do not justify separate species status for this group.

NEW GENERA

De Lajudie et al. (1994) renamed part of the genus *Rhizobium* when proposing the new species *S. saheli* and *S. teranga*, because *saheli* and *teranga* constitute a separate phylogenetic branch together with *fredii* and *meliloti* and this branch has a 16S ribosomal sequence similarity with the *R. leguminosarum* cluster of 95-96%. This value is comparable with those used to separatre out other genera within the proteobacteria. Because ***Sinorhizobium*** was validly proposed to contain the species *fredii* as the type species by Chen et al. (1988), the genus name *Sinorhizobium* has nomenclatural priority whenever a new group containing the species *fredii* is created. Therefore, the species *meliloti*, a widely studied species under its former name, had for phylogenetic reasons to be transferred to *Sinorhizobium*. However, the new name does not appeal now to the scientific community and calls for caution when introducing new names. This does not mean that naming taxonomic groups should be avoided, just that new names should be a consequence of detailed and critical scientific work followed by discussions to find an agreeable name. We live in a changing world and this extends to rhizobial nomenclature.

Since strains on the *R. loti* phylogenetic branch show only a mean of 93% similarity with other rhizobial branches, this branch merits distinction as a new genus. The name "***Mesorhizobium***" was proposed and discussed for this branch and the proposal was supported.

One group of bradyrhizobia is phylogenetically distinct from *B. japonicum* types I and IA was proposed as a possible new genus.This branch contains the type strains of *B. elkanii* and shows 97% similarity with the type strain of *B. japonicum*. It is thus on the boarderline for generic separation from the standpoint of phylogeny.

R. galegae is the only rhizobial group that seems to be closer to agrobacteria (*A. tumefaciens*, *A. vitis* and *A. rubi*) than to other rhizobia. Nevertheless, as there is a long evolutionary distance also to the agrobacteria, some results point at a mosaic structure for its ribosomal sequences and new agrobacterium-like rhizobia were reported at this meeting (e.g. Nick et al., this volume), renaming *R. galegae* would be premature.

CONCLUDING REMARKS

In order to understand the huge biodiversity in the rhizobial world, we need to set high standards for taxonomic work with rhizobia and agrobacteria. By doing so, taxonomy can, in addition to naming new species and genera, serve ecological studies as well as evolutionary ones. After initial screening by methods such as RFLP of ribosomal sequences, determination of protein profiles in SDS-PAGE, rep-PCR, RAPD analysis, analysis of total cellular fatty acids, membrane DNA hybridization, multilocus enzyme electrophoresis or numerical taxonomy, DNA relatedness and the sequences of ribosomal genes should be critically determined and the taxa described with respect to phenotypic traits. Appropriate reference strains and DNA sequences should be used when making comparisons. Cultures are available free of charge from the USDA-ARS National Rhizobium Culture Collection headed by Peter van Berkum (e-mail: PBERKUM@ASRR.ARSUSDA.GOV). Since there are a number of different tree drawing algoritms available when constructing phylogenies, it would be desirable to develop and use new methods for validating trees. Tree drawing can be highly subjective but overall similarity values can be used in cases where reliable nodes are hard to obtain. Laboratories are encouraged to exchange strains and methods in order to achieve these goals.

To facilitate communication, the newsgroup for biological nitrogen fixation in BIONET will be used for newsletters and discussions. Information is also available from the secretary of the subcommittee (e-mail:
KRISTINA.LINDSTROM@HELSINKI.FI).

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